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(57) Abstract

A DNA construct comprising (i) means of expression of a coding sequence in a tumour cell and (ii) a said coding sequence encoding a cytokine. The said means for expression may provide for specific expression selectively in tumour cells, particularly melanoma cells, and pancreatic, breast, colonic and prostatic tumour cells and the cytokine is at least one of interleukin-2, interleukin-4, macrophage colony stimulating factor, interferon-γ, tumour necrosis factor and interleukin-7.

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TUMOUR THERAPY

The present invention relates to the therapy of tumours, particularly melanomas.

Biological therapy of cancer, based upon the adoptive transfer of modified immune cells, seeks to exploit *in vivo* specificity to deliver recombinant proteins directly to the tumour mass (Parmiani *et al* (1992) *Trends Exp. Clin. Med.* **2**, 412-419; Rosenberg (1992) *J. Clin. Oncol.* **10**, 180-100). However, this approach involves removal of cells from the patient followed by their *in vitro* manipulation and replacement *in vivo*. Proposed vaccination experiments using genetically modified tumour cells also require a similar period of passage *in vitro* during which time the neoplastic cells may significantly alter their immunological properties or growth characteristics (Rosenberg (1992) *loc. cit.*; Roemer & Friedmann (1992) *Eur. J. Biochem.* **208**, 211-225; Pardoll (1992) *Curr. Opin. Immunol.* **4**, 619-623); Fearon *et al* (1990) *Cell* **60**, 397-403.

There is experimental evidence that the expression of cytokines in tumour cells (following transfection with cytokine cDNA *in vitro*) leads to rejection of otherwise tumourigenic doses of tumour cells and, in some cases, can immunise animals against established diseases when the transfected cells are injected into the animal. Cytokines shown to have this effect include interleukin-2, interleukin-4, interferon-γ, tumour necrosis factor and interleukin-7. This information is summarised in Pardoll (1992) *Curr. Opinion Immunol.* **4**, 619-623.

CD28-positive T cell responses, and immune responses mediated by T cells, may be regulated by the B7 antigen as described in WO 92/00092. Also, tumour rejection after direct costimulation of $CD8^+$ T cells by B7-

transfected melanoma cells is described in Townsend & Allison (1993) *Science* **259**, 368-370.

Malignant melanoma represents a cancer the growth and dissemination of which may be altered significantly by immunological manipulation. Many melanomas synthesise the pigment melanin, which is otherwise produced almost exclusively by melanocytes (Hearing & Tsukamoto (1991) *FASEB J.* **5**, 2902-2909) and indeed several workers have proposed utilising the melanin synthetic pathway for chemotherapeutic intervention (Riley (1991) *Eur. J. Cancer* **27**, 1172-1179; Link & Carpenter (1992) *Cancer Res.* **52**, 4385-4390).

The tyrosinase and TRP-1 genes both encode proteins which play key roles in the synthesis of the pigment melanin, a specific product of melanocytic cells. Our aim has been to utilise the 5′ ends of the tyrosinase and tyrosinase-related protein (TRP-1) genes to confer tissue specificity of expression on genes cloned downstream of these promoter elements for therapeutic purposes.

A number of other groups already have shown that tissue specificity of expression resides within the 5′ sequences of these genes (eg Bradl, M. *et al* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 164-168; Jackson, I.J. *et al* (1991) *Nucleic Acids Res.* **19**, 3799-3804). However we have confirmed and expanded these findings and used the promoters of these genes for therapeutic purposes.

Prostate-specific antigen (PSA) is one of the major protein constituents of the human prostate secretion. It has become a useful marker for the detection and monitoring of prostate cancer. Other groups have characterised the gene encoding PSA and have identified the promoter

region which directs the prostate-specific expression of PSA (Lundwall (1989) *Biochem. Biophys. Res. Comm.* **161**, 1151-1159; Riegman *et al* (1989) *Biochem. Biophys. Res. Comm.* **159**, 95-102; Brawer (1991) *Acta Oncol.* **30**, 161-168).

Carcinoembryonic antigen (CEA) is a widely used tumour marker, especially in the surveillance of colonic cancer patients. Although CEA is also present in some normal tissues, it is apparently expressed at higher levels in tumorous tissues than in corresponding normal tissues. The complete gene encoding CEA has been cloned and its promoter region analysed. A CEA gene promoter construct, containing approximately 400 nucleotides upstream from the translational start, showed nine times higher activity in the adenocarcinoma cell line SW303, compared with the HeLa cell line. This indicates that *cis*-acting sequences which convey cell type specific expression are contained within this region (Schrewe *et al* (1990) *Mol. Cell. Biol.* **10**, 2738-2748).

The c-*erb*B-2 gene and promoter have been characterised previously and the gene product has been shown to be over-expressed in tumour cell lines (Kraus *et al* (1987) *EMBO J.* **6**, 605-610).

The mucin gene, MUC1, contains 5′ flanking sequences which are able to direct expression selectively in breast and pancreatic cell lines, but not in non-epithelial cell lines as taught in WO 91/09867.

Summary of the Invention

One aspect of the invention provides a DNA construct comprising (i) means for expression of a coding sequence in a tumour cell and (ii) a said coding sequence encoding a cytokine.

Expression of the cytokine in the tumour cells is believed to stimulate attack by T cells, especially LAK cells. Such T cells will then destroy not only the primary tumour but also any secondary (metastatic) growths.

The tumour may be a melanoma, or a tumour of the breast, colon, brain, pancreas, bladder, skin, prostate, stomach, oesophagus or liver, for example. Preferably, it is a melanoma.

Advantageously, the said means for expression provides for specific expression selectively in tumour cells. Otherwise, the T cells may attack normal cells and/or the germ line may be altered.

By "specific expression selectively in tumour cells" we mean that the expression is usefully higher (for example 2X, 5X, 10X or at least 20X higher) in tumour cells compared to the expression in non-tumour cells. It will be appreciated by those skilled in the art that tumour selective expression may be derived from tissue-specific expression where the tumour rapidly grows from a specific tissue type. Alternatively, highly specific delivery of a non-specific expression construct may be adequate. Known means such as targeted liposomes (carrying anti-tumour-marker antibodies) and viruses, including retroviruses, may be employed.

The constructs of the invention may be introduced into the tumour cells by any convenient method, for example methods involving retroviruses, so that the construct is inserted into the genome of the tumour cell. For example, in Kuriyama *et al* (1991) *Cell Struc. and Func.* **16**, 503-510 purified retroviruses are administered. Retroviruses provide a potential means of selectively infecting cancer cells because they can only integrate into the genome of dividing cells; most normal cells surrounding cancers are in a quiescent, non-receptive stage of cell growth. Retroviral DNA

constructs which contain a promoter segment and a cytokine coding sequence may be made using methods well known in the art. To produce active retrovirus from such a construct it is usual to use an ecotropic psi2 packaging cell line grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS). Transfection of the cell line is conveniently by calcium phosphate co-precipitation, and stable transformants are selected by addition of G418 to a final concentration of 1 mg/ml (assuming the retroviral construct contains a *neo*^{R} gene). Independent colonies are isolated and expanded and the culture supernatant removed, filtered through a 0.45 μm pore-size filter and stored at -70°. For the introduction of the retrovirus into the tumour cells, it is convenient to inject directly retroviral supernatant to which 10 μg/ml Polybrene has been added. For tumours exceeding 10 mm in diameter it is appropriate to inject between 0.1 ml and 1 ml of retroviral supernatant; preferably 0.5 ml. Alternatively, as described in Culver *et al* (1992) *Science* **256**, 1550-1552, cells which produce retroviruses are injected into the tumour. The retrovirus-producing cells so introduced are engineered to actively produce retroviral vector particles so that continuous productions of the vector occurred within the tumour mass *in situ*. Thus, proliferating tumour cells can be successfully transduced *in vivo* if mixed with retroviral vector-producing cells. Other methods involve simple delivery of the construct into the cell for expression therein either for a limited time or, following integration into the genome, for a longer time. An example of the latter approach includes (preferably tumour-cell-targeted) liposomes (Nãssander *et al* (1992) *Cancer Res.* 52, 646-653).

Immunoliposomes (antibody-directed liposomes) are especially useful in targeting to cancer cell types which over-express a cell surface protein for which antibodies are available. In relation to the present invention, antibodies directed towards tumour cell antigens such as CEA and PSA

are preferred. For the preparation of immuno-liposomes MPB-PE (N-[4-(p-maleimidophenyl)butyryl]-phosphatidylethanolamine) is synthesised according to the method of Martin & Papahadjopoulos (1982) *J. Biol. Chem.* **257**, 286-288. MPB-PE is incorporated into the liposomal bilayers to allow a covalent coupling of the antibody, or fragment thereof, to the liposomal surface. The liposome is conveniently loaded with the DNA construct of the invention for delivery to the target cells, for example, by forming the said liposomes in a solution of the DNA construct, followed by sequential extrusion through polycarbonate membrane filters with 0.6 μm and 0.2 μm pore size under nitrogen pressures up to 0.8 MPa. After extrusion, entrapped DNA construct is separated from free DNA construct by ultracentrifugation at 80 000 x g for 45 min. Freshly prepared MPB-PE-liposomes in deoxygenated buffer are mixed with freshly prepared antibody (or fragment thereof) and the coupling reactions are carried out in a nitrogen atmosphere at 4°C under constant end over end rotation overnight. The immunoliposomes are separated from unconjugated antibodies by ultracentrifugation at 80 000 x g for 45 min. Immunoliposomes may be injected intraperitoneally or directly into the tumour.

It will be appreciated that monoclonal antibodies or other molecules that bind to tumour cell surface antigens are useful in targeting the DNA construct of the invention.

Monoclonal antibodies which will bind to many of these antigens are already known but in any case, with today's techniques in relation to monoclonal antibody technology, antibodies can be prepared to most antigens. The antigen-binding portion may be a part of an antibody (for example a Fab fragment) or a synthetic antibody fragment (for example a single chain Fv fragment [ScFv]). Suitable monoclonal antibodies to

selected antigens may be prepared by known techniques, for example those disclosed in *"Monoclonal Antibodies: A manual of techniques"*, H Zola (CRC Press, 1988) and in *"Monoclonal Hybridoma Antibodies: Techniques and Applications"*, J G R Hurrell (CRC Press, 1982).

Chimaeric antibodies are discussed by Neuberger *et al* (1988, *8th International Biotechnology Symposium* Part 2, 792-799).

Suitably prepared non-human antibodies can be "humanized" in known ways, for example by inserting the CDR regions of mouse antibodies into the framework of human antibodies. Such "humanized" antibodies, or fragments thereof, are preferred as they may give rise to a lower anti-antibody reaction than rodent antibodies.

The variable heavy (V_H) and variable light (V_L) domains of the antibody are involved in antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by "humanisation" of rodent antibodies. Variable domains of rodent origin may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parented antibody (Morrison *et al* (1984) *Proc. Natl. Acad. Sci. USA* 81, 6851-6855).

That antigenic specificity is conferred by variable domains and is independent of the constant domains is known from experiments involving the bacterial expression of antibody fragments, all containing one or more variable domains. These molecules include Fab-like molecules (Better *et al* (1988) *Science* **240**, 1041); Fv molecules (Skerra *et al* (1988) *Science* **240**, 1038); single-chain Fv (ScFv) molecules where the V_H and V_L partner domains are linked via a flexible oligopeptide (Bird *et al* (1988) *Science* **242**, 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**,

5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward *et al* (1989) *Nature* **341**, 544). A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) *Nature* **349**, 293-299.

By "ScFv molecules" we mean molecules wherein the V_H and V_L partner domains are linked via a flexible oligopeptide.

The advantages of using antibody fragments, rather than whole antibodies, are several-fold. The smaller size of the fragments may lead to improved pharmacological properties, such as better penetration of solid tissue. Effector functions of whole antibodies, such as complement binding, are removed. Fab, Fv, ScFv and dAb antibody fragments can all be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of the said fragments.

Whole antibodies, and $F(ab')_2$ fragments are "bivalent". By "bivalent" we mean that the said antibodies and $F(ab')_2$ fragments have two antigen combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent, having only one antigen combining sites.

Other molecules immunologically reactive with the target cell surface molecule are also useful in this aspect of the invention and include, for example minimal recognition units (MRU) and complementarity determining regions.

Other methods of delivery include adenoviruses carrying external DNA via an antibody-polylysine bridge (see Curiel *Prog. Med. Virol.* **40**, 1-18) and transferrin-polycation conjugates as carriers (Wagner *et al* (1990) *Proc.*

Natl. Acad. Sci. USA **87**, 3410-3414). In the first of these methods a polycation-antibody complex is formed with the DNA construct of the invention, wherein the antibody is specific for either wild-type adenovirus or a variant adenovirus in which a new epitope has been introduced which binds the antibody. The polycation moiety binds the DNA via electrostatic interactions with the phosphate backbone. The adenovirus, because it contains unaltered fibre and pentos proteins, is internalized into the cell and carries into the cell with it the DNA construct of the invention. It is preferred if the polycation is polylysine.

In the second of these methods, a high-efficiency nucleic acid delivery system that uses receptor-mediated endocytosis to carry DNA macromolecules into cells is employed. This is accomplished by conjugating the iron-transport protein transferrin to polycations that bind nucleic acids. Human transferrin, or the chicken homologue conalbumin, or combinations thereof is covalently linked to the small DNA-binding protein protamine or to polylysines of various sizes through a disulfide linkage. These modified transferrin molecules maintain their ability to bind their cognate receptor and to mediate efficient iron transport into the cell. The transferrin-polycation molecules form electrophoretically stable complexes with DNA constructs of the invention independent of nucleic acid size (from short oligonucleotides to DNA of 21 kilobase pairs). When complexes of transferrin-polycation and the DNA constructs of the invention are supplied to the tumour cells, a high level of expression from the construct in the cells is expected.

High-efficiency receptor-mediated delivery of the DNA constructs of the invention using the endosome-disruption activity of defective or chemically inactivated adenovirus particles produced by the methods of Cotten *et al* (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6094-6098 may also be used. This

approach appears to rely on the fact that adenoviruses are adapted to allow release of their DNA from an endosome without passage through the lysosome, and in the presence of, for example transferrin linked to the DNA construct of the invention, the DNA construct is taken up by the cell by the same route as the adenovirus particle.

It may be desirable to locally perfuse a tumour with the delivery vehicle (for example the retrovirus) for a period of time.

In one embodiment of the invention the said means for expression provides for specific expression selectively in melanoma cells or in melanoma cells and melanocytes. In this embodiment the said means for expression is a promoter or an analogue or part thereof forming part of a gene expressed substantially exclusively in the melanin synthesis pathway. Examples of such promoters include the tyrosinase gene promoter and the tyrosinase-related protein (TRP-1) gene promoter.

By "promoter" we mean that region of DNA which controls, at least to a substantial extent, the transcription of the coding region associated with that region of DNA.

In a further embodiment of the invention the said means for expression provides for specific expression selectively in prostate cancer cells or prostate cancer cells and prostate cells. In this embodiment the said means for expression is a promoter or an analogue or part thereof forming part of a gene expressed substantially exclusively in prostate cancer or prostate cells. An example of such a promoter is the prostate-specific antigen (PSA) gene promoter.

In a still further embodiment of the invention the said means for

expression provides for specific expression selectively in colonic cancer cells, or colonic cancer cells and colon cells. In this embodiment the said means for expression is a promoter or an analogue or part thereof forming part of a gene expressed substantially exclusively in colon cancer or colon cells. An example of such a promoter is the carcinoembryonic antigen (CEA) gene promoter.

In another embodiment of the invention the said means for expression is provided by the promoter region of the c-*erb*B2-gene.

In this embodiment the constructs comprising the c-*erb*B2 gene promoter fused to the cytokine coding sequence may be usefully delivered to breast tumours. The c-*erb*B3 gene promoter may also be used.

In yet another embodiment the said means for expression is provided by the promoter region of the MUC1 gene.

In this embodiment pancreatic or breast tumours may usefully receive the constructs comprising MUC1 gene promoter fused to the cytokine coding sequence.

DNA sequences encompassing the promoter sequences useful in the invention are given in the sequence listing.

The cytokine is preferably interleukin-2 or interleukin-4 or macrophage colony stimulating factor. Other cytokines may, however, be used, for example interferon-γ, tumour necrosis factor, and interleukin-7. Nucleotide coding sequences for these are known and are given in the sequence listing.

The promoter is joined to the cytokine coding region and placed in a suitable vector system for propagation. The skilled person can use the information given below containing the promoter DNA sequences and coding sequences of some of the cytokines useful in the invention to make suitable constructs. For example, a knowledge of the DNA sequences provides information on where restriction enzyme will cleave the said DNA molecules and allows oligonucleotide primers to be designed for PCR amplification and site-directed mutagenesis.

The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the recombinant DNA construct of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the propagation of the DNA construct, which can then be recovered.

The vectors usually include a procaryotic replicon, such as the ColE1 *ori*, for propagation in a procaryote, even if the vector is to be used for expression in other, non-procaryotic, cell types.

It is preferred if the host cell is *E. coli*.

A variety of methods have been developed to operatively link DNA to vectors via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3′-single-stranded termini with their 3′-5′-exonucleolytic activities, and fill in recessed 3′-ends with their polymerizing activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

DNA fragments with complementary cohesive termini are readily joined

together by ligation using methods known in the art and described in Sambrook *et al* (1989) *Molecular Cloning, A laboratory manual*, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

A desirable way to modify the promoter fragment, vector or coding region to be fused in the DNA construct is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491.

In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

The present invention also relates to a host cell transformed with a polynucleotide vector construct of the present invention. The host cell for propagating the DNA construct can be either procaryotic or eucaryotic. Bacterial cells are preferred host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343).

Transformation of appropriate cell hosts with a DNA construct of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of bacterial, especially *E. coli* host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2110 and Sambrook *et al* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor

Laboratory, Cold Spring Harbor, NY.

Successfully transformed cells, ie cells that contain a DNA construct of the present invention, can be identified by well known techniques. For example, cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* **98**, 503 or Berent *et al* (1985) *Biotech.* **3**, 208 or by isolating the plasmid vector DNA and then digesting the said plasmid appropriate restriction enzymes that give diagnostic DNA fragments that can be separated and sized by gel electrophoresis.

The DNA construct of the invention is purified from the host cell using well known methods.

For example, plasmid vector DNA can be prepared on a large scale from cleaved lysates by banding in a CsCl gradient according to the methods of Clewell & Helinski (1970) *Biochemistry* **9**, 4428-4440 and Clewell (1972) *J. Bacteriol.* **110**, 667-676. Plasmid DNA extracted in this way can be freed from CsCl by dialysis against sterile, pyrogen-free buffer through Visking tubing or by size-exclusion chromatography.

Alternatively, plasmid DNA may be purified from cleared lysates using ion-exchange chromatography, for example those supplied by Qiagen. Hydroxyapatite column chromatography may also be used.

Preferably, naked DNA is injected in the tumour, for example at a dose of 0.1 ng to 1.0 mg vector DNA cm^{-3} of tumour, preferably about 0.1-10 $\mu g\ cm^{-3}$ vector DNA. The DNA may be circular or linear. Linear DNA may be obtained from circular DNA by cleavage with an appropriate restriction enzyme.

By "appropriate restriction enzyme" we mean one that does not cleave the DNA within the promoter region or cytokine coding region.

At present, it is most preferable to use 1.0 μg of DNA per cm^3 of tumour in a volume of 100 μl. The DNA may be dissolved in phosphate-buffered saline (PBS), or it may be used as a precipitate with calcium phosphate. Of course, other suitable buffers or carriers may usefully be employed. The expression of the said DNA in the tumour may be analysed by reverse transcriptase-PCR (that is, the messenger RNA expressed from the DNA in the tumour is isolated, converted into complementary DNA (cDNA) using the enzyme reverse transcriptase, and the resultant cDNA is amplified using the polymerase chain reaction and may be detected radiolabelling or staining), or by northern blot analysis or by RNase protection assays.

Such injection may be repeated at hourly, daily or weekly intervals.

Uptake of naked DNA may depend on the three-dimensional growing mass of tumour so, although it is preferred that the tumour to be treated is melanoma, a prostate tumour, or a colon tumour or a pancreatic tumour, or a breast tumour, it may be any solid tumour.

It is most preferred if substantially all cells in the tumour take up DNA and express the cytokine, but it is not essential for a useful clinical effect, as the antitumour effect of the cytokine is not limited to the tumour cell expressing the cytokine but will occur in non-transfected cells within the tumour and at secondary (metastatic) sites. Thus, if 5%, preferably 25%, more preferably 50% and most preferably substantially 100% of the tumour cells express the cytokine a clinically useful effect may be seen.

It is desirable to express a plurality of cytokine coding sequences in a tumour cell, or to express a plurality of cytokine coding sequences in a tumour wherein each cytokine coding sequence is present in a separate DNA construct. It is preferable if the different cytokines, expressed by the plurality of coding sequences, stimulate different effector cells of the immune system.

In one embodiment, each of the coding sequences of the plurality are directly joined to a means for expression in a tumour cell but are contained within the same DNA construct. Thus, once the DNA is introduced into the tumour, every cell that takes up the DNA may express all of the cytokine coding sequences in the plurality.

In a further embodiment, a plurality of DNA constructs is introduced into the tumour, each construct of the plurality comprises a means for expression of a coding sequence in a tumour cell and a coding sequence encoding a different cytokine. In this embodiment it is possible to vary the proportion of cytokine coding sequences in the plurality.

The components of the plurality comprise two or more of coding sequences encoding interleukin-2, interleukin-4, macrophage colony stimulating factor, interferon-γ, tumour necrosis factor and interleukin-7. The ratio of any two of the said coding sequences in the plurality may be, one to another, 100:1, 10:1 or 1:1.

Thus, a particular plurality of coding sequences useful in the invention is interleukin-2:interleukin-4:macrophage colony stimulating factor in a molar ratio of 1:1:1. This particular combination of coding sequences will express a plurality of cytokines useful in attracting cytotoxic T cells, eosinophils and macrophages to the tumour, and to secondary (metastatic)

sites. All of these cell types have been shown to have anti-tumour activity.

It is preferred that the means of expressing each coding sequence in the plurality is a tumour specific promoter.

It is preferred that the plurality of DNA constructs is injected directly into the tumour.

It is further preferred that the tumour into which the DNA construct is injected directly is a melanoma, breast cancer, prostate cancer or colon cancer.

It is desirable to treat melanoma with a DNA construct wherein the means of expressing is the tyrosinase promoter.

In a further embodiment it is preferred if the B-cell accessory molecule B7 antigen is co-expressed with the cytokine in the tumour or tumour cell. B7 binds CD28 on T-cells and stimulates the activity of T-cells against tumours as is described in WO 92/00092.

The cDNA encoding the B7 antigen molecule can be obtained using the method described by Freeman *et al* (1989) *J. Immunol.* **143**, 2714-2722 incorporated herein by reference and the nucleotide and predicted amino acid sequence can be obtained therefrom. The nucleotide sequence of B7 cDNA is given as SEQ ID No 23.

The term "fragment" as used herein means a portion of the amino acid sequence corresponding to the B7 antigen. For example, a fragment of the B7 antigen useful in the method of the present invention is a

polypeptide containing a portion of the amino acid sequence corresponding to the extracellular portion of the B7 antigen, ie the DNA enclodin g amino acid residues from position 1 to 215 of the sequence corresponding to the B7 antigen described by Freeman *et al, supra*.

Complementary cDNA sequences encoding the amino acid sequence corresponding to the B7 antigen or fragments or derivatives thereof can be synthesised by the polymerase chain reaction (see US Patent No 4,683,202) using primers derived from the published sequence of the antigen (Freeman *et al*, *supra*). These cDNA sequences can then be assembled into a vector so that the expression of the B7 antigen is driven by a means for expression in the tumour cell.

It is preferred if the means for expression is a tumour-specific promoter.

It is further preferred if the promoter is the tyrosinase or TRP-1 promoter.

It is preferred if the tumour is melanoma.

The techniques for assembling and expressing DNA encoding the amino acid sequences corresponding to B7 antigen and the cytokines useful in the invention, eg synthesis of oligonucleotides, PCR, transforming cells, constructing vectors and the like are well-established in the art, and most practitioners are familiar with the standard resource materials for specific conditions and procedures. However, the following paragraphs are provided for convenience and notation of modifications where necessary, and may serve as a guideline.

Complementary cDNA clones containing DNA encoding B7 proteins are obtained to provide DNA for assembling into the DNA constructs for use

in the methods of the invention. Alternatively, cDNA clones may be prepared from RNA obtained from cells expressing B7 antigen or the cytokines based on knowledge of the published sequences for these proteins using standard procedures. Published sequences for the cDNAs are given as SEQ ID Nos.

The cDNA is amplified using the polymerase chain reaction ("PCR") technique (see US Patent Nos. 4,683,195 and 4,683,202 to Mullis *et al* and Mullis & Faloona (1987) *Methods Enzymol.* **154**, 335-350) using synthetic oligonucleotides encoding the sequences of the proteins as primers. PCR is then used to adapt the fragments for ligation to the DNA encoding the promoter fragments and to expression plasmid DNA to form cloning and expression plasmids.

It is desirable to express a single cytokine coding sequence or a plurality of cytokine coding sequences in a tumour cell, in combination with the B7 coding sequence, or to express a cytokine coding sequence in a tumour in combination with a B7 coding sequence wherein the cytokine coding sequence and the B7 coding sequence are present in a separate DNA construct. It is preferable if the different cytokines, expressed by the plurality of coding sequences, stimulate different effector cells of the immune system.

In one embodiment, each of the coding sequences of the plurality of cytokines or B7 coding sequence are directly joined to a means for expression in a tumour cell but are contained within the same DNA construct. Thus, once the DNA is introduced into the tumour, every cell that takes up the DNA may express all of the cytokine coding sequences in the plurality and the B7 coding sequence.

In a further embodiment, a plurality of DNA constructs is introduced into the tumour, each construct of the plurality comprises a means for expression of a coding sequence in a tumour cell or a coding sequence encoding a different cytokine or B7 molecule. In this embodiment it is possible to vary the proportion of cytokine coding sequences and B7 molecules introduced into the tumour.

It will be appreciated by one skilled in the art that the same or different cytokine or B7 coding sequence may be expressed in the tumour cell from separate DNA constructs or that the said coding sequences may be expressed in the tumour cell from the same DNA construct wherein each coding sequence has an independent means for expression or that the said coding sequences may be expressed in the tumour cell from the same DNA construct wherein each coding sequence has the same means for expression. In the latter case the coding sequences for a cytokine or a B7 may be fused such that a fusion polypeptide is made; it is preferred if a linker joins the polypeptides in the fusion that is cleaved in the environment of the tumour cell to release the active cytokine or B7.

When melanoma is to be treated by the DNA constructs comprising a gene promoter from a melanin synthesis pathway gene such as tyrosinase, it is desirable if the patient to be treated is not black.

It is further preferred if the patient to be so treated is fair-skinned.

In a further aspect of the invention the DNA constructs are used in conjunction with chemotherapy. Thus, the DNA construct, or a plurality of such constructs, may be administered at the same time as, preceding or after treatment with chemotherapeutic agents.

Chemotherapeutic agents useful in this aspect of the invention include cisplatin, dacarbazine, tamoxifen, nitrosoureas including carmusine (BCNU), vinca alkaloids, melphalan, doxorubicin, adriamycin, etoposide, 5-fluorouracil and other generally used agents.

These are listed in the table:

TABLE: CHEMOTHERAPEUTIC AGENTS

Class	Type of Agent	Nonproprietary Names (Other Names)
Alkylating Agents	Nitrogen Mustards	Mechlorethamine (HN_2)
		Cyclophosphamide Ifosfamide
		Melphalan (L-sarcolysin)
		Chlorambucil
	Ethylenimines and Methylmelamines	Hexamethylmela-mine
		Thiotepa
	Alkyl Sulfonates	Busulfan
	Nitrosoureas	Carmustine (BCNU)
		Lomustine (CCNU)
		Semustine (methyl-CCNU)
		Streptozocin (streptozotocin)
	Triazenes	Decarbazine (DTIC; dimethyltria-zenoimi-dazolecarbox-amide)

Class	Type of Agent	Nonproprietary Names (Other Names)
Antimetabolites	Folic Acid Analogs	Methotrexate (amethopterin)
	Pyrimidine Analogs	Fluorouracil (5-fluorouracil; 5-FU) Floxuridine (fluorode-oxyuridine; FUdR)
		Cytarabine (cytosine arabinoside)
Antimetabolites continued	Purine Analogs and Related Inhibitors	Mercaptopurine (6-mercaptopurine; 6-MP)
		Thioguanine (6-thioguanine; TG)
		Pentostatin (2′-deoxycoformycin)

Class	Type of Agent	Nonproprietary Names (Other Names)
Natural Products	Vinca Alkaloids	Vinblastine (VLB)
		Vincristine
	Epipodophyl-lotoxins	Etoposide Teniposide
	Antibiotics	Dactinomycin (actinomycin D)
		Daunorubicin (daunomycin; rubidomycin)
		Doxorubicin
		Bleomycin
		Plicamycin (mithramycin)
		Mitomycin (mitomycin C)
	Enzymes	L-Asparaginase
	Biological Response Modifiers	Interferon alfa
Miscellaneous Agents	Platinum Coordination Complexes	Cisplatin (*cis*-DDP) Carboplatin
	Anthracenedione	Mitoxantrone
	Substituted Urea	Hydroxyurea
	Methyl Hydrazine Derivative	Procarbazine (N-methylhydrazine, MIH)
	Adrenocortical Suppressant	Mitotane (*o,p'*-DDD)
		Aminoglutethimide

It is preferred if the DNA construct or the plurality of constructs expresses interleukin-2 which will facilitate the substantial destruction of the vasculature and promote the action of the chemotherapeutic agent.

Further aspects of the invention provide a composition comprising a construct of the invention and means for selectively delivering it to a tumour and a method of treating a tumour and/or ameliorating metastasis therefrom comprising delivering into cells of the tumour a construct of the invention.

The invention will now be described with reference to the following Examples and Figures wherein:

Figure 1 shows the tissue specific expression cassettes using the tyrosinase and the TRP-1 gene promoters;

Figure 2 shows the relative activity of tyrosinase and TRP-1 promoters in murine B16.F1 melanoma and NIH 3T3 cells;

Figure 3 shows the retroviral vector pBabe Puro (Tyr-β-Gal).

Figure 4 shows the c-*erb*B-2/CAT construct of Example 5.

Figure 5 shows the result of a comparison of activity of the construct of Example 5 in two cell lines: T47D, which is a breast carcinoma cell line with base line c-*erb*B-2 expression, and ZR75-1, which is a breast carcinoma cell line with elevated c-*erb*B-2 expression.

SEQ ID No 1 shows the nucleotide sequence of the CEA gene including the promoter region.

SEQ ID No 2 shows the sequence of the PSA gene including the promoter region.

Figure 6 shows the 5′ flanking sequence with 71 bp of transcribed sequence of the human MUC1 gene (SEQ ID No 3). The TATA box (boxed) and transcriptional start site (+1) are indicated. The sequence (-787 to +71) covers the region required for maximum transcription of the reporter gene (-743 to +33).

Figure 7 shows the DNA sequence of the human c-*erb*B-2 5′ region as determined by Hudson *et al* (1990) *J. Biol. Chem.* 265, 4389-4393 (SEQ ID No 4).

Figure 8 shows the DNA sequence of the human c-*erb*B-3 5′ region (SEQ ID No 5) and the predicted amino acid sequence of the first exon (SEQ ID No 6).

SEQ ID No 7 shows the DNA sequence of the tyrosinase promoter.

SEQ ID No 8 shows the DNA sequence of the TRP-1 promoter.

SEQ ID No 9 shows the DNA gene sequence encoding interleukin-2 (IL-2); the cDNA sequence is readily derived from the positions of the exons.

SEQ ID No 10 shows the cDNA sequence encoding interleukin-4 (IL-4).

SEQ ID No 11 shows the cDNA sequence encoding interleukin-7 (IL-7).

SEQ ID No 12 shows the cDNA sequence encoding tumour necrosis factor (TNF).

SEQ ID No 21 shows the cDNA sequence encoding interferon-gamma (IFN-γ).

SEQ ID No 22 shows the cDNA sequence encoding human granulocyte macrophage colony stimulating factor GM-CSF.

SEQ ID No 23 shows the B7 cDNA sequence.

The following information is useful to the person skilled in the art to identify coding regions and promoter sequences for use in the invention. Journal references and EMBL database accession numbers are given.

SEQ ID No 1

ID HSCEA01 standard; DNA; PRI; 3281 BP; AC M59255; M31966; DE Human carcinoembryonic antigen (CEA) gene, complete cds; KW carcinoembryonic antigen; OS Homo sapiens (human); OC Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia; OC Theria; Eutheria; Primates; Haplorhini; Catarrhini; Hominidae; RN [1]; RP 1-3281; RA. Schrewe H., Thompson J., Bona M., Hefta L.J., Maruya A.,; RA Hassauer M., Shively J.E., von Kleist S., Zimmermann W; RT "Cloning of the complete gene for carcinoembryonic antigen:; RT Analysis of its promoter indicates a region conveying cell; RT type-specific expression"; RL Mol. Cell. Biol. 10:2738-2748(1990); FH Key Location/Qualifiers; FH; FT sig_peptide join(1769..1832,2725..2762); FT /gene="CEA"; FT exon 1659..1832; FT /number=1 /gene="CEA" /codon_start=1659; FT exon 2725..3084; FT /number=2 /gene="CEA" /codon_start=2725; SQ Sequence 3281 BP; 847 A; 953 C; 871 G; 610 T; 0 other; CC

SEQ ID No 21

ID HSIFNGAMM standard; RNA; PRI; 1011 BP; AC M26683; DT 23-NOV-1989 (Rel. 21, Created); DT 26-MAY-1992 (Rel. 32, Last updated, Version 5); DE Human interferon gamma (IFN-gamma) mRNA, complete cds; KW interferon gamma; type II; OS Homo sapiens (human); OC Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia; OC Theria; Eutheria; Primates; Haplorhini; Catarrhini; Hominidae; RN. [1]; RP 1-1011; RA Fan X., Stark G.R., Bloom B.R; RT "molecular cloning of a gene selectively induced by gamma; RT interferon from human macrophage cell line u937"; RL Mol. Cell. Biol. 9:1922-1928(1989); FH Key Location/Qualifiers; FH; FT CDS 15..131; FT /product="interferon gamma" /gene="IFN-gamma"; FT /codon_start=1; FT polyA_signal 971..976; FT /gene="IFN-gamma"; SQ Sequence 1011 BP; 301 A; 236 C; 184 G; 290 T; 0 other;

SEQ ID No 2

ID HSPSAA standard; DNA; PRI; 7130 BP; AC M27274; DT 23-APR-1990 (Rel. 23, Last updated, Version 1); DT 02-FEB-1990 (Rel. 22, Created); DE Human prostate-specific antigen gene, complete cds; KW Alu repetitive element; kallikrein; prostate specific antigen; OS Homo sapiens (human); OC Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia; OC Theria; Eutheria; Primates; Haplorhini; Catarrhini; Hominidae; RN [1]; RP 1-7130; RA Lundwall A; RT. "Characterization of the gene for prostate-specific antigen, a; RT human glandular kallikrein"; RL Biochem. Biophys. Res. Commun. 161:1151-1159(1989); DR SWISS-PROT; P07288; PROS$HUMAN; FH Key Location/Qualifiers; FH; FT CDS 675..720; FT /note="prostate-specific antigen, exon 1; FT /nomgen="APS"

/map="19q13.3-qter"; FT /hgml_locus_uid="LN0098S""; FT intron 721..1958; FT /note="PSA intron A"; FT CDS 1959..2118; FT /note="prostate-specific antigen, exon 2"; FT intron 2119..3755; FT /note="PSA intron B"; FT repeat_region 2583..2935; FT /note="Alu repeat"; FT CDS 3756..4042; FT /note="prostate-specific antigen, exon 3"; FT intron 4043..4185; FT /note="PSA intron C"; FT CDS 4186..4322; FT /note="prostate-specific antigen, exon 4"; FT intron 4323..5698; FT /note="PSA intron D"; FT CDS 5699..5854; FT /note="prostate-specific antigen, exon 5"; SQ Sequence 7130 BP; 1530 A; 2024 C; 1867 G; 1709 T; 0 other;

SEQ ID No 8

ID MMTRP15 standard; DNA; ROD; 1236 BP; AC X59513; DT 26-JUL-1991 (Rel. 28, Created); DT 26-JUL-1991 (Rel. 28, Last updated, Version 2); DE Mouse 5' end of TRP1 gene for tyrosinase-related protein-1; KW TRP1 gene; tyrosinase; tyrosinase-related protein-1; OS Mus musculus (mouse); OC Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia; OC Theria; Eutheria; Rodentia; Myomorpha; Muridae; Murinae; RN. [1]; RA Jackson I.J., Chambers D.M., Budd P.S., Johnson R; "The tyrosinase-related protein-1 gene has a structure and promoter sequence very different from tyrosinase.";Nucleic Acids Res. 19:3799-3804(1991) SQ Sequence 1236 BP; 357 A; 234 C; 282 G; 363 T; 0 other;

SEQ ID No 22

ID HSCSFGMA standard; DNA; PRI; 3194 BP; AC M13207; DT 07-JUN-1987 (Rel. 12, Created); DT 24-DEC-1990 (Rel. 26, Last updated, Version 2); DE Human granulocyte-macrophage

colony-stimulating factor (hGM-CSF); DE gene, complete cds; KW granulocyte-macrophage colony stimulating factor; OS Homo sapiens (human); OC Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia; OC Theria; Eutheria; Primates; Haplorhini; Catarrhini; Hominidae; RN [1]; RP 1-3194; RA Kaushansky K., O'Hara P.J., Berkner K., Segal G.M., Hagen F.S.,; RA Adamson J.W; RT "Genomic cloning, characterization, and multilineage; RT growth-promoting activity of human granulocyte-macrophage; RT colony-stimulating factor"; RL Proc. Natl. Acad. Sci. U.S.A. 83:3101-3105(1986); RN [2]; RP 1-3194; RA Kaushansky K; RT; RL Unpublished; DR CPGISLE; HSCSFGMA; Release pre-1.0; DR SWISS-PROT; P04141; CSF2_HUMAN; SQ Sequence 3194 BP; 700 A; 859 C; 945 G; 690 T; 0 other; CC

SEQ ID No 9

ID HSIL21 standard; DNA; PRI; 5737 BP; AC J00264; DT 29-JUL-1991 (Rel. 28, Created); DT 29-JUL-1991 (Rel. 28, Last updated, Version 1); DE Human interleukin 2 (Il-2) gene, complete coding sequence; KW immune response gene; interleukin; interleukin 2; lymphokine; KW T-cell; T-cell growth factor; OS Homo sapiens (human); OC Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia; OC Theria; Eutheria; Primates; Haplorhini; Catarrhini; Hominidae; RN [1]; RP 431-624, 715-774, 3068-3211, 5057-5443; RA Maeda S., Nishino N., Obaru K., Mita S., Nomiyama H., Shimada K.,; RA Fujimoto K., Teranishi T., Hirano T., Onoue K; RT "Cloning of interleukin 2 mRNAs from human tonsils"; RL Biochem. Biophys. Res. Commun. 115:1040-1047(1983); RN CC Key Location/Qualifiers; FH; FT CDS join (478..624,715..774,3068..3211,5057..5167); SQ Sequence 5737 BP; 1995 A; 932 C; 922 G; 1888 T; 0 other; CC; ID HSIL4 standard; RNA; PRI; 614 BP; AC M13982; DT 07-JUN-1987 (Rel. 12, Created); DT

03-SEP-1992 (Rel. 33, Last updated, Version 2);

SEQ ID No 10

KW interleukin; OS Homo sapiens (human); OC Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia; OC Theria; Eutheria; Primates; Haplorhini; Catarrhini; Hominidae; RN [1]; RP 1-614; RA Yokota T., Otsuka T., Mosmann T., Banchereau J., DeFrance T.,; RA Blanchard D., De Vries J.E., Lee F., Arai K.i."Isolation and characterization of a human interleukin cDNA clone homologous to mouse B-cell stimulatory factor 1, that expresses B-cell- and T-cell-stimulating activities"Proc. Natl. Acad. Sci. U.S.A. 83:5894-5898(1986). ; DR SWISS-PROT; P05112; IL4_HUMAN; FH Key Location/Qualifiers; FH; FT mRNA <1..614; FT /note="IL-4 mRNA"; FT CDS 64..524; FT /note="interleukin 4" /gene="IL4" /partial; FT sig_peptide 64..135; FT /note="interleukin 4 signal peptide"; FT mat_peptide 136..522; FT /note="interleukin 4 mature peptide"; SQ Sequence 614 BP; 174 A; 150 C; 129 G; 161 T; 0 other;

SEQ ID No 11

ID HSIL7A standard; RNA; PRI; 1589 BP; AC J04156; DT 22-APR-1989 (Rel. 19, Created); DT 06-JUL-1989 (Rel. 20, Last updated, Version 1); DE Human interleukin 7 (IL-7) mRNA, complete cds; KW interleukin; interleukin 7; OS Homo sapiens (human); OC Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia; OC Theria; Eutheria; Primates; Haplorhini; Catarrhini; Hominidae; RN [1]; RP 1-1589; RA Goodwin R.G., Lupton S., Schmierer A., Hjerrild K.J., Jerzy R.,; RA Clevenger W., Gillis S., Cosman D., Namen A.E; RT "Human interleukin 7: Molecular cloning and growth factor activity; RT

on human and murine B-lineage cells"; RL Proc. Natl. Acad. Sci. U.S.A. 86:302-306(1989); DR SWISS-PROT; P13232; IL7_HUMAN; CC Draft entry and computer-readable sequence [1] kindly submitted by; CC R.Goodwin, 05-JAN-1989; FH Key Location/Qualifiers; FH; FT mRNA <1..1589; FT /note="interleukin 7 mRNA"; FT CDS 385..918; FT /note="interleukin 7 precursor"; FT CDS 385..459; FT /note="interleukin 7 signal peptide"; FT CDS 460..915; FT /note="interleukin 7"; SQ Sequence 1589 BP; 532 A; 284 C; 339 G; 434 T; 0 other;

SEQ ID No 12

Human tumour necrosis factor mRNA; ID HSTNFAA standard; RNA; PRI; 1585 BP; AC M10988; DT 16-JUL-1988 (Rel. 16, Created); DT 02-SEP-1992 (Rel. 33, Last updated, Version 2); DE Human tumor necrosis factor (TNF) mRNA; KW ; OS Homo sapiens (human); OC Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia; OC Theria; Eutheria; Primates; Haplorhini; Catarrhini; Hominidae; RN [1]; RP 1-1585; RA Wang A.M., Creasey A.A., Ladner M.B., Lin L.S., Strickler J.,; RA Van Arsdell J.N., Yamamoto R., Mark D.F; RT "Molecular cloning of the complementary DNA for human tumor; RT necrosis factor"; RL Science 228:149-154(1985); DR SWISS-PROT; P01375; TNFA_HUMAN; FH Key Location/Qualifiers; FH; FT CDS 86..787; FT /note="tumor necrosis factor" /gene="TNFA"; FT /codon_start=1; SQ Sequence 1585 BP; 352 A; 473 C; 389 G; 371 T; 0 other; CC

SEQ ID No 7

ID MMTYR1 standard; DNA; ROD; 4758 BP; AC D00439; DT

14-FEB-1991 (Rel. 27, Created); DT 14-FEB-1991 (Rel. 27, Last updated, Version 1); DE Mouse tyrosinase gene, 5'flank and exon 1; KW melanin; melanocyte; monooxygenase; tyrosinase; OS Mus musculus (mouse); OC Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia; OC Theria; Eutheria; Rodentia; Myomorpha; Muridae; Murinae; RN [1]; RP 2481-3363; RA ; RN [2]; RP 1-4758; RA Yamamoto H., Takeuchi S., Kudo T., Sato C., Takeuchi T; RT "Melanin production in cultured albino melanocytes transfected; RT with mouse tyrosinase cDNA"; RL Jpn. J. Genet. 64:121-135(1989); FH Key Location/Qualifiers; FH; FT misc_signal 2004..2008; FT /note="putative CAT box"; FT misc_signal 2128..2133; FT /note="putative CAT box"; FT misc_signal 2140..2146; FT /note="putative TATA box"; FT misc_signal 2264..2268; FT /note="putative CAT box"; FT misc_signal 2272..2279; FT /note="putative TATA box"; FT misc_signal 2286..2289; FT /note="putative CAT box"; FT misc_signal 2434..2440; FT /note="putative TATA box"; FT misc_feature 2465..2466; FT /note="CAP sites"; FT CDS 2545..>3363; FT /note="tyrosinase gene, exon 1" /partial; SQ Sequence 4758 BP; 1550 A; 859 C; 878 G; 1465 T; 6 other; CC

SEQ ID No 23

; ID HSIGB7 standard; RNA; PRI; 1491 BP. ; AC M27533; ; DT 23-APR-1990 (Rel. 23, Created) ; DT 23-APR-1990 (Rel. 23, Last updated, Version 1) ; DE Human Ig rearranged B7 protein mRNA VC1-region, complete cds. ; KW constant region; immunoglobulin; variable region. ; OS Homo sapiens (human) ; OC Eukaryota; Animalia; Metazoa; Chordata; Vertebrata; Mammalia; ; OC Theria; Eutheria; Primates; Haplorhini; Catarrhini; Hominidae. ; RN [1] ; RP 1-1491 ; RA Freeman G.J., Freedman A.S., Segil J.M., Lee G., Whitman J.F., ; RA

Nadler L.M.; ; RT "B7, a new member of the Ig superfamily with unique expression on ; RT activated and neoplastic B cells"; ; RL J. Immunol. 143:2714-2722(1989). ; CC Draft entry and computer readable copy of sequence [1] kindly ; CC provided by G.J. Freeman, 08-SEP-1989. ; FH Key Location/Qualifiers ; FH ; FT CDS 318..1184 ; FT /note="transmembrane protein B1 precursor" ; FT CDS 318..395 ; FT /note="transmembrane protein B1 signal ; FT peptide" ; FT CDS 396..1181 ; FT /note="transmembrane protein B1" ; SQ Sequence 1491 BP; 419 A; 343 C; 311 G; 418 T; 0 other; ; CC

Example 1: Demonstration of tissue specificity of 5′ sequences of murine tyrosinase and TRP-1 genes.

A 2.5kb fragment from the 5′ end of the tyrosinase gene was generated by PCR from genomic DNA of the B16 melanoma line. The oligonucleotides used (Pair 1: 5′-CGGAATTTCATGCCCCAGTTGAC-AACATAG-3′, SEQ ID No 13; 5′-CACTCGAGAACTTTTTCTCCT-TTAGATCATACAA-3′, SEQ ID No 14) were derived from the murine sequence published by Yamamoto *et al* (1989) *Jpn. J. Genet.* **64**, 121-135. Shorter 5′ sequences were generated also using oligonucleotides matched from the Yamamoto paper (Pair 2: 5′CGGGAATTCATGCCCCAGTTGACAACATAG-3′, SEQ ID No 15; 5′-GAGCTCGAGTGTCACAGACTTCTTTTCCA-3, SEQ ID No 16; Pair 3: 5′-AAACGAATTCCATCCAGTAAGTCCATTACT-3′, SEQ ID No 17; 5′-GAGCTCGAGTGTCACAGACTTCTTTC-3′, SEQ ID No 18). The 769bp fragment of the tyrosinase gene extends from position -815 to position -46 in the promoter. A 4.0kb fragment of 5′ sequence of the TRP-1 gene was provided by Dr I.J. Jackson, MRC Genetics Unit, Edinburgh and from this a 1.4kb fragment was derived by *Xba*I-*Sal*I digestion. The promoter sequence at the 5′ of TRP-1 gene may be

obtained following the methods described in Jackson *et al* (1991) *Nucl. Acids Res.* **19**, 3799-3804.

These 5′ sequences, and the SV40 promoter as a control, were inserted upstream of the β-galactosidase gene in the vector pNASS (obtained from Clontech Ltd) as indicated in Figure 1.

Figure 1 shows (A) pNASSβ, a promoterless mammalian expression vector described by MacGregor & Caskey (1989) *Nucl. Acids Res.* **17**, 2365. Three unique restriction sites allow cloning of promoter sequences upstream of an expression cassette containing the SV40 splice donor/acceptor sequence (sd/sa), the β-galactosidase gene and the SV40 polyadenylation sequence. SV40 β-Gal contains the SV40 early viral promoter (from the pBabe Puro vector, as described by Morgenstern & Land (1990) *Nucl. Acids Res.* **18**, 3596, cloned into pNASSβ. (B) 2496 bp (Tyr-β-Gal 1) or 769 bp (Tyr-β-Gal 2) fragments of the mouse tyrosinase promoter (Yamamoto *et al* (1989) *Jap. J. Genet.* **64**, 121-135) were generated by PCR from genomic DNA of the B16.F1 melanoma cell line and cloned into the *Eco*RI and *Xho*I restriction sites of pNASSβ. (C) The plasmids TRP-1-β-Gal 1 and 2 were a gift from I. Jackson and contain 4 kbp and 1.4 kbp of the TRP-1 promoter (Jackson *et al* (1991) *Nucl. Acids Res.* **19**, 3798-3804) upstream of the β-galactosidase gene and the SV40 polyadenylation sequence. The different constructs were transfected into a variety of murine and human cells of melanocytic and non-melanocytic origin, including B16 melanoma cells or NIH 3T3 fibroblasts and subsequent β-galactosidase activity was measured 72-96 hours after transfection both by fluorometric assay, using 4-methylumbelliferyl-β-D-galactoside (MUG) as substrate, and by histochemical analysis using X-gal as substrate. By both assays the various tyrosinase and TRP-1 promoter containing 5′ sequences were

shown to drive β-galactosidase activity in a murine melanocyte (Mel-ab) line and the B16 melanoma and the human melanoma lines SK23, HMB-2, Mel 8, TXM13, T8 and SS3. No activity was observed in the murine 3T3 or L cell lines or the human HeLa, LS174T, HT29, HOS, SW620 and HUVEC lines, none of which are of melanocytic origin (see Figure 2 and Table 1).

Figure 2 shows the relative activity of tyrosinase and TRP-1 promoters in murine B16.F1 melanoma and NIH 3T3 cells. Cells were transfected with 10 μg of the appropriate plasmid DNA using the calcium phosphate method. 72-96 hours after the calcium phosphate precipitate had been washed away the cells were analysed for expression of β-galactosidase using the quantitative MUG assay. Data are expressed as mean of triplicate values $\pm$ SD. The data presented are representative of four similar experiments.

In contrast, the SV40 promoter was able to direct expression of the reporter gene to high levels in both cell types.

Table 1

Species	Cell Line	Tissue Type	Expression of: Tyr-β-Gal	TRP-1-β-Gal
Mouse	Melab	Melanocyte	+	+
	B16	Melanoma	+	+
	1735P	Melanoma	+	+
	1735 C19	Melanoma	+	+
	NIH3T3	Fibroblast	-	-
	L cells	Fibroblast	-	-
	AKR	T cell leukaemia	-	-
	Colo 26	Colon	-	-
Rat	Gli C	Glioma	-	-
Hamster	BHK-21	Kidney	-	-
Human	SK23	Melanoma	+	-
	HMB2	Melanoma	+	+
	5S3	Melanoma	+	+
	Mel 8	Melanoma	+	+
	Mel 17	Melanoma	+	+
	TXM13	Melanoma	+	+
	T8	Melanoma	+	+
	A375M	Melanoma	+	+
	VUP	Ocular Melanoma	-	-
	DX3	Melanoma	-	-
	HeLa	Cervical carcinoma	-	-
	HOS	Osteosarcoma	-	-
	HT29	Colorectal carcinoma	-	-
	SW620	Colorectal carcinoma	-	-
	LS174T	Colorectal carcinoma	-	-
	HUVEC	Endothelium	-	-

<u>Footnote to Table 1.</u> Cell type specificity of expression of β-galactosidase from Tyrosinase and TRP-1 promoters. Each cell line indicated was transfected with 10 μg of plasmid DNA of Tyr-β-Gal 1 and 2, TRP-1-β-Gal 1 and 2. pNASS-β and SV40-β-Gal were used in each case as a negative and positive control for transfection. Expression of β-galactosidase was scored as positive (+) if several cells stained blue 96 hours after transfection; a cell line was scored as negative (-) if no blue cells were observed after transfection and if the quantitative MUG assay showed no expression above background levels (transfection with pNASS-

β).

These results confirm and extend the reports of other groups showing excellent tissue specificity of gene expression in melanocytic cells of either murine or human origin when the 5′ promoter regions of either the tyrosinase or TRP-1 gene are utilised.

<u>Example 2: Materials and methods pertaining to the other Examples</u>.

Construction of Expression Plasmids and Retroviral Vectors. Subcloning was carried out via standard recombination DNA techniques (Sambrook *et al* (1989) *Molecular cloning, a laboratory manual*, Cold Spring Harbor Laboratory Press, NY, USA). Restriction endonuclease enzymes were supplied by Northumbria Biologicals (NBL, Cramlington, UK) and Taq polymerase was supplied by Stratech (Luton, UK). Oligonucleotides, synthesised on an Applied Biosystems 380B and purified by denaturing acrylamide electrophoresis, were provided by the Oligonucleotide Synthesis Laboratory, ICRF Clare Hall, South Mimms, UK. Polymerase chain reaction (PCR) amplification of DNA fragments was carried out on a Techne PHC-2 Thermocycler and reaction mixes were prepared in a hood separate from normal areas of DNA handling. Amplified DNA sequences were subcloned into the PCR II vector (Invitrogen; British Biotechnology Products Ltd, Oxford, UK) and their identities were confirmed by restriction endonuclease mapping. The correct fragments were then shuttled from PCR II into the appropriate expression plasmid.

Cell Culture. All cell lines used in this study were checked routinely and found to be free of mycoplasma infection. Apart from Melab cells which were cultured in medium supplemented as described previously (Burrows

et al (1991) *Cancer Res.* 51, 4768-4775) the lines were grown in Eagle's minimal essential medium supplemented with 10% (v/v) fetal calf serum and 4 mM L-glutamine. HUVEC (Human umbilical vein endothelial cells) were maintained in Medium 199 (Gibco-Biocult Ltd, Paisley, Scotland) supplemented with Earle's salts, 20% (v/v) fetal calf serum, endothelial cell growth supplement (0.12 mg/ml) 0.09 mg.ml heparin and glutamine. Cultures were maintained at 37°C in a humidified atmosphere of 90% air/10% CO_2.

DNA Transfection. 10^6 adherent cells were transfected with 10 μg of plasmid DNA by calcium phosphate co-precipitation using the Profection method (Promega, Madison, WI) according to the manufacturer's instructions. 24 hours after the application of the precipitate to the tissue culture medium, cells were washed three times in serum-free medium and incubated in normal medium for 72-96 hours when they were stained for β-galactosidase expression.

Intra-Tumoral Injection of DNA. 1-1.5 x 10^5 tumour cells of either the B16 F1 murine melanoma or the Colo 26 colon carcinoma were injected subcutaneously in 100 μl inoculum volumes into the flank region of syngeneic mice (C57 for B16 F1, Balb/C for Colo 26). Ten days later the animals were anaesthetised by halothane inhalation (ICI Pharmaceuticals, Macclesfield, UK), the tumours, approximately 4 mm in diameter, were located by palpation and injected with 1 μg DNA in 100 μl volumes of either PBS or as calcium phosphate precipitates via a 27-gauge needle.

Quantitative Assay for β-Galactosidase Expression. Transfected cells were assayed for enzyme activity by the technique of MacGregor *et al* (1991) *Methods in Molecular Biology* 7, 217-235 (Ed., E.J. Murray) Humana Press Inc, Clifton, NJ, USA. Briefly cells were resuspended in

Z buffer (60 mM $Na_2PO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 1 mM $MgSO_4.7H_2O$) at 10^7 cells per ml. 105 μl of this cell suspension were dispensed per well of a microtiter plate and 15 μl of 1% Triton X-100 were added to each well to give a final concentration of 0.1%. After 10 minutes at room temperature, 30 μl of 3 mM methylumbelliferyl-β-D-galactoside (MUG) (Sigma, Poole, UK) in Z buffer were added to each well and the reaction was allowed to proceed for 90 minutes at 37°C. 75 μl of 300 mM glycine, 15 mM EDTA, pH 11.2 were added to stop the reaction. Fluorescence was measured on a microtiter dish fluorescence reader (excitation at 350 nm and emission read at 450 nm).

Cells expressing β-galactosidase convert the MUG substrate, a non-fluorescent galactoside analogue, to the fluorescent molecule 4-methylumbelliferone.

Histochemical Detection of β-Galactosidase-expressing Cells. 72-96 hours following DNA transfection, adherent cells were washed once in phosphate buffered saline (PBS) and fixed for 10 minutes at 4°C with 3.8% formaldehyde in PBS. The fixative was removed by three washes with PBS and the cells were then incubated with X-gal solution [5-bromo-4-chloro-3-indoyl-β-galactopyranoside (Sigma) at 40 mg/ml in dimethylformamide was diluted to 1 mg/ml in 5 mM $K_3Fe(CN)_6$; 5 mM $K_4Fe(CN)_6.3H_2O$; 2 mM $MgCl_2$; 0.01% sodium deoxycholate; 0.2% NP40. All solutions were prepared using glass] at 37°C for at least 4 hours according to published techniques (Bondi *et al* (1982) *Histochem.* 76, 153-158). After staining the X-gal solution was removed, the cells were washed three times in PBS and the cells were inspected under a light microscope. Cells expressing the β-galactosidase gene hydrolyse the chromogenic substrate X-gal to give the blue dye bromochloroindole. Control untransfected cells also were stained to assess the background

endogenous β-gal staining.

Detecting of β-Galactosidase-expressing Tumour Cells. 2, 4, 6 or 10 days after injection of DNA into the tumours, animals were killed by CO_2 inhalation, their tumours were excised, minced to 1 mm cubes with scalpels and pushed through a stainless steel sieve with a 5 ml syringe plunger, into culture medium. An aliquot of the resulting cell suspension was spun onto a glass microscope slide using a cytospin centrifuge. Slides were air-dried then fixed for 5 minutes in 3.8% formaldehyde in PBS. The cells were rinsed in PBS and incubated overnight in X-gal stain before being inspected under a light microscope for the presence of blue cells.

Generation of Recombinant Retrovirus Stocks. The AM 12 packaging cell line (Markowitz *et al* (1988) *Virol.* **167**, 400-406) containing the packaging constructs for Moloney Leukaemia Virus was transfected with 10 μg of retroviral plasmid DNA using the calcium phosphate co-precipitation method. 48 hours following transfection the cells were split into puromycin (Sigma) selection medium (1 μg/ml) and surviving colonies were selected and pooled two weeks later. Virus was harvested from these producer cells by exposing fresh medium to 5 x 10^6 cells on a 90 mm plate and harvesting the medium 16 hours later. The medium was filtered through a 0.45 μm filter (Nalge (UK) Ltd, Rotherwas, England) to remove cell debris and was then used to infect target cells. The target cells were split 24 hours earlier to a density of 10^5 cells per 90 mm plate. Polybrene (Aldrich, Gillingham, Dorset) was added to the viral supernatant to 4 μg/ml to enhance virus-cell surface interactions and the target cells were exposed to 1 ml of viral supernatant for 2.5 hours at 37°C. 8 ml of normal growth medium were added to the plate and the infected cells were grown for a further 72-96 hours before being stained for expression of β-galactosidase.

Example 3: Preparation of tyrosinase promoter- or TRP-1 promoter-driven expression vectors containing cytokine cDNA's.

The pBCMGNeo-mIL-2 vector was provided by Dr P. Frost, University of Texas, Houston and is described in *Eur. J. Immunol.* **18**, 97-194 (1988), although other vectors are suitable. This vector had been used to transfect B16 melanoma cells (a non-cell-type-specific approach) and IL-2 producing cells had been selected (Fearon *et al* (1990) *Cell* **60**, 397-403). The HCMV promoter of this vector was removed by *Xba*1-*Sal*1 digestion and replaced with the 1.4kb *Xba*1-*Sal*1 fragment of TRP-1 5′ sequences or the 780bp tyrosinase 5′ sequence fragment generated by Pair 3 oligonucleotides. These constructs were transfected into murine B16 melanoma cells or 3T3 fibroblasts. For the TRP-1 - IL2 construct a total of 60 puromycin-resistant clones were isolated and screened by ELISA for IL-2 production (Genzyme Ltd). Clones were characterised as high (≥ 960 pg/ml), intermediate (150-960 pg/ml) and low (≤ 150 pg/ml) expressers. Of the 60 clones, 13 clones were found to be producing and secreting measurable quantities of IL-2 while ten clones of 3T3 cells and four pooled bulk populations of 3T3 did not contain any cells expressing detectable amounts of IL-2 activity. These results show that the tissue-specific promoter, TRP-1, is able to drive expression of a cytokine cDNA in an appropriate cell type. Repeated analysis over a 6-8 week culture period showed that the observed phenotype is stable.

Alternatively, the IL-2 coding sequence can be incorporated into a tyrosinase promoter vector as follows:

The murine IL-2 cDNA is PCR amplified from pBCMGNeo mIL-2 using the primers GCGGCCGCGCATGTACAGCATGCAGCTCGCA (SEQ ID No 19) and GCGGCCGCTAAATAAATAGAGAGCCTTATG (SEQ ID

No 20).

The PCR fragment is cloned into the vector PCRII (available from Invitrogen) and then excised from the PCRII vector using *Not*I digestion. The *Not*I fragment is cloned into the *Not*I site of Tyr-β-Gal-1 (described in Example 1) in place of the β-galactosidase gene. This produces Tyr IL-2 with a 2494 bp promoter from the tyrosinase gene driving expression of IL-2.

B16 clones have been injected into groups of syngeneic C57 mice. To date only the cell clone selected for drug resistance, ie lacking IL-2 expression, is forming progressively growing tumours in these animals. The IL-2 secreting B16 cells are not forming palpable tumours and, if they do develop, are clearly growing at a slower rate *in vivo*.

In addition to the cells secreting IL-2, IL-2 expression is assessed using RT-PCR wherein RNA is isolated, primers such as oligo dT used to prime synthesis of cDNA from the mRNA using reverse transcriptase and the level of IL-2 RNA estimated by amplifying with IL-2-specific oligonucleotides.

We have placed cDNA for IL-4 (bought from British Biotechnology Ltd) downstream of both promoter sequence but the construct may utilise any cytokine gene (eg GM-CSF, TNF, IFN), be combined with the HSV tk gene for ganciclovir selection, or may utilise cDNAs encoding for genes which might stimulate the immune response (eg MHC antigens, MAGE (melanoma antigens) etc). This procedure allows targeted expression of the requisite gene to the cell type of interest, ie melanocyte-derived cells. Replacement of the tyrosinase or TRP-1 promoter sequences with sequences which are expressed by other tumour types in a specific fashion

(eg 5′ promoter sequences of the CEA gene for colorectal tumours, 5′ sequences of prostate secreted antigen for prostatic tumours) permits targeted expression of similar genes to other tumour types.

<u>Example 4: Introduction of tissue specific promoter-driven genes into target cells *in vivo*.</u>

There are two main routes of delivery:-

1) Retroviral delivery
2) Direct delivery

Incorporation into a Retroviral Vector. The ability of the melanocyte-specific promoters to function after delivery via a retroviral vector was examined because retroviral-mediated gene delivery is a promising route for delivery of gene therapy *in vivo* (Miller (1992) *Nature* 357, 455-460). The retroviral vector pBabe Tyr-β-Gal was constructed from the pBabe Puro vector (Morgenstern & Land (1990) *Nucl. Acids Res.* **18**, 3587-3596) (Figure 3). Here β-galactosidase is expressed from the 769 bp tyrosinase promoter fragment of Tyr-β-Gal 2 inserted into pBabe Puro in the opposite orientation to the direction of expression of the viral mRNA driven from the Moloney Leukaemia Virus (MLV) Long Terminal Repeat (LTR).

Following transfection of the vector into the AM12 amphotropic packaging cell line, recombinant retroviral particles were used to infect either B16 or NIH 3T3 cells. 72-96 hours following infection, expression of the β-galactosidase gene was observed preferentially in B16 cells relative to the NIH 3T3 target cells by both histochemical and fluorimetric assays.

These results demonstrate that the tyrosinase and TRP-1 promoters can confer tissue specificity of expression upon an heterologous gene in both human and murine melanocyte-derived cell lines when delivered in the context of a retroviral vector.

Experiments on route 2 have yielded interesting results. Syngeneic C57/BL mice were injected s.c. in the flank region with $1x10^5$ B16 cells and the animals were monitored until a tumour of approximately 0.4 x 0.4 cm had developed. Similar Colo tumours were established in Balb-C mice. At this time a single injection of 1.0 μg of the tyrosinase promoter/pNASS DNA was inoculated in 100 μl volumes directly into the centre of the tumour either as 'naked' DNA or as calcium phosphate-coprecipitated material. Similarly, pNASS-β and TRP-β-Gal-2 DNA was inoculated. At varying times thereafter, for example at 2, 4, 6 or 10 days, mice were killed, and the tumours were removed and snap-frozen. Cryostat sections of these tumours were stained for β-galactosidase activity. Protein expression, manifest by the detection of bright blue cells, was clearly apparent in the majority of the injected tumours. The Tyr-β-Gal 2 construct caused the gradual accumulation of positive blue cells in the injected B16 tumours over the ten day period of examination; whereas the same construct injected into the non-melanocytic Colo 26 tumours produced no blue staining. Similar results were obtained in three independent replicate experiments and from these it was apparent that:- (1) the promoterless, control pNASS β construct produced no blue cells in either Colo 26 or B16 tumours; (2) there was a gradual increase in the proportion of blue cells in the positive groups over the 10 day period of examination (10 days was the last time-point examined because of increasing tumour burden) up to an estimated 10-15% of cells (3) no qualitative or quantitative difference was obvious between the tyrosinase or TRP-1 promoter elements or between material injected as naked DNA

or as a $CaPO_4$-precipitate. Frozen sections of B16 tumours stained 10 days after DNA injection showed similar results. Interestingly the only blue-staining tissue, apart from the neoplastic cells, was confined to the base of the hair follicles and thus, presumably, indicated transduction of normal melanocytes.

These results show that direct gene transfer may be accomplished by intra-tumoural injections. Morphological assessment of the sections indicated that the blue cells were restricted to areas occupied by neoplastic tissue, which is presumed to reflect the tissue specificity conferred by the 5′ tyrosinase or TRP-1 gene sequence.

These experiments suggest that direct injections permit good levels of expression of introduced genes. The activity produced may be altered by modification of the introduced DNA (eg incorporation in liposomes, use of different precipitating material, variation in route of delivery). Taken in combination our results indicate that placing therapeutic genes under control of tissue-specific promoter regions may restrict expression to cells of a specific lineage. This could be important both for safety/specificity purposes and would permit the refinement of what otherwise may be a fairly non-specific event. The utilisation of a cytokine gene has been shown to induce modifications in subsequent tumour behaviour. Direct delivery of DNA via an intratumoural injection has been shown to produce high levels of expression of the introduced gene suggesting that such promoter-restricted expression may be further limited to the target cells by the simple expedient of targeting inoculation. The use of genes encoding for proteins capable of eliciting a subsequent systemic response may permit this method to be used for disseminated, rather than localised, neoplastic disease.

Example 5: c-*erb*B-2 promoter and reporter enzyme

Reporter enzyme gene. The bacterial chloramphenicol acetyl transferase (CAT) gene was obtained from Promega as the "pCAT-basic" vector.

The CAT reporter system is designed to allow sensitive and rapid testing for eukaryotic transcriptional regulatory sequences. This reporter system relies on the linkage of genomic DNA fragments containing putative regulatory sequences to the chloramphenicol acetyltransferase (CAT) reporter gene. Transcriptional effects upon the CAT reporter gene are detected after transfection into cultured cells. Since CAT is a bacterial gene, levels of CAT enzyme activity in crude cell extracts can be quickly and easily assayed with little or no background from endogenous cellular gene activity. The pCAT-Basic plasmid lacks eukaryotic promoter and enhancer sequences. This allows the researcher maximum flexibility in cloning any putative regulatory sequences into the convenient multiple cloning sites. Expression of CAT activity in cells transfected with this plasmid is dependent on insertion of a functional promoter upstream from the CAT gene. Enhancer elements can be inserted upstream from the promoter or at the *Bam*HI site downstream from the CAT gene. Sequences to be tested for transcriptional activity can be cloned into the following unique sites located immediately upstream from the CAT gene: *Xba*I, *Acc*I, *Sal*I, *Pst*I, *Sph*I and *Hind*III. Enhancer elements can be cloned separately into the *Bam*HI site downstream from the CAT transcriptional unit. The vector also contains the gene for ampicillin resistance.

Promoter. The human c-*erb*B-2 promoter has been cloned to -500 by two groups (Ishi *et al* (1987) *Proc Natl Acad Sci USA* **84**, 4374-4378; Tal *et al* (1987) *Mol Cell Biol* **7**, 2597-2601) and to -1500 by a third group

(Hudson *et al* (1990a) *J Biol Chem* 265, 4389-4393). We have taken oligonucleotides to 30b regions around +40 and -500 and, using PCR against human genomic DNA, recovered a 540bp fragment representing the c-*erb*B-2 proximal promoter. Using oligos to -1000 and -500 we then "PCRed" out a further 500bp representing the c-*erb*B-2 distal promoter. The two promoter regions were fused at the *Sma*I site at -500 and the full promoter cloned upstream of the CAT gene to generate a reporter plasmid for assaying c-*erb*B-2 promoter activity in cell lines *in vitro*. Further constructs were made by either deleting 5′ regions of the promoter using convenient restriction enzyme sites, or using PCR technology, to generate a series of promoter deletion mutants linked to CAT 3′ end always +40; 5′ ends as follows: -1000, -500, -400, -300, -213, -177, -100; (Figure 1).

Construction of c-*erb*B-2 plasmid. The c-*erb*B-2 promoter was incorporated in the pCAT-basic plasmid to give the plasmid shown in Figure 1 by digesting the plasmid with *Xba*I and then filling the ends with Klenow fragment to create a blunt-ended vector suitable for cloning the blunt-ended PCR products.

The CAT activity from the various promoter constructs was compared to baseline activity from the promoterless CAT parent plasmid by calcium phosphate mediated DNA transfection into a number of different breast cell lines. Immortalised normal and tumour lines which have low endogenous c-*erb*B-2 expression showed little activity of the c-*erb*B-2 promoter, ie all the reporter constructs containing c-*erb*B-2 sequences generated no more CAT activity than the promoterless control plasmid. This result makes it unlikely that c-*erb*B-2 expression is actively repressed in these cell lines (by a tumour suppressor-like activity).

Example 6: Promoter region of the carcinoembryonic antigen gene

The CEA gene is cloned using standard methods as described by Schrewe *et al* (1990) *Mol. Cell. Biol.* **10**, 2738-2748 and sequenced using the dideoxy chain termination method of Sanger *et al* (1980) *J. Mol. Biol.* **143**, 161-178.

To define the actual portion of the 5′ untranslated region which is required for the promoter activity of the CEA gene, we carried out functional tests by placing restriction endonuclease fragments of various lengths from the putative promoter regions of both genes upstream of the CAT reporter gene and assaying for CAT activity in a transient transfection assay in two different human cell lines. For this purpose, we chose the CEA-producing adenocarcinoma cell line SW403 and, as a negative control, the HeLa cell line. The CEA promoter constructs showed an enhanced expression of the CAT gene in SW403 cells, which was nine times greater than in HeLa cells, when the shortest construct was used. It appears that *cis* regulatory sequences, which are responsible for this enhancement, along with a functional transcription initiator, are both present within the first 424 nucleotides upstream of the translational start. It is also interesting that longer CEA constructs are approximately 50% less active in HeLa cells than is the shortest construct. A possible explanation for this phenomenon is that a silencer region could exist between nucleotides -424 and -832 upstream from the translational start, which reduces the activities in both cell lines through interaction with common *trans*-acting regulatory factors. Such silencer sequences have indeed been described for other genes.

Thus, the promoter of the CEA gene is useful for expressing cytokines, according to the methods of the invention, in colon tumours.

As found here for CEA, a number of other eucaryotic genes have also been reported which do not contain obvious TATA boxes. The promoters of such genes can be divided into two classes. The members of the first class are G+C rich and are found primarily in housekeeping genes. These promoters usually contain several transcription initiation sites spread over a fairly large region, as well as potential binding sites for Spl. The members of the second class are not G+C rich, are not constitutively active, but are regulated during differentiation or development and initiate transcription at only one or a few tightly clustered start sites. Included in this class are a number of genes that are regulated during mammalian immunodifferentiation, eg the T-cell receptor β-chain genes and the V_{preB} gene, as well as some *Drosophila* homeotic genes. The CEA gene shows a closer resemblance to this latter group, because its promoter is not obviously G+C rich, it contains no identifiable Spl-binding sites, it reveals only a few tightly clustered start sites, and, most importantly, it is not constitutively expressed.

Figure 6 shows the nucleotide sequence from the promoter region of CEA compared with the promoter region of the non-specific cross-reacting antigen gene (NCA) and the CGM1 gene. The numbers indicate the distance in nucleotides from the initiation codon for each gene. Gaps have been introduced to allow optimal alignment. Identical nucleotides are indicated by dots. The cluster of transcriptional start sites determined for CEA and NCA by S1 nuclease assays are indicated by arrows.

Example 7: Promoter region of the prostate-specific antigen gene

The PSA gene is cloned using standard methods as described by Riegman *et al* (1989) *Biochem. Biophys. Res. Comm.* **159**, 95-102 and Lundwall (1989) *Biochem. Biophys. Res. Comm.* **161**, 1151-1159 and sequenced

using the dideoxy chain termination method of Sanger *et al* (1980) *J. Mol. Biol.* **143**, 161-178.

The sequence of the promoter region of PSA gene, compared to that of the hGK-1 gene, is shown in Figure 7. Dots represent identical nucleotides. Putative transcriptional regulatory elements are boxed.

PSA is expressed at a high level in the prostate; hGK-1, a human kallikrein-like gene, is expressed at lower level in the prostate.

The differences in nucleotide sequence between the PSA and hGK-1 promoters are probably important determinants in prostate-specific gene expression.

Thus, the promoter of the PSA gene is useful for expressing cytokines, according to the method of the invention, in prostate tumours.

Example 8: Promoter region of the MUC1 gene

The mucin gene, MUC1, is selectively expressed in breast and pancreatic cell lines but not in non-epithelial cell lines. The promoter region for this gene may be obtained by the methods taught in WO 91/09867.

The 5′ sequences flanking the human MUC1 gene are analyzed for their ability to direct expression of a reporter gene (the chloramphenical transferase gene, CAT) in cell lines which normally express or do not express the MUC1 gene. A construct containing 2.9 kb of MUC1 5′ flanking sequence shows expression of CAT in breast and pancreatic cell lines but not in the non-epithelial cell lines HT 1080, SK23 and HTB96. Deletion analysis shows that maximum expression was obtained in ZR-75

(breast cancer line) and HPAP (pancreatic cancer line) with only 743 bp of 5′ flanking sequence. Sequences within 1.6 kb of the transcriptional start site showed enhancing activity in a vector carrying an enhancerless SV40 promoter. Analysis of proximal 5′ sequences in a promoterless CAT vector carrying the SV40 enhancer shows that sequences between -60 and -150 were crucial for tissue specific expression. An Spl site at -99/-90 and an E-box (E-MUC1) at -84/-64 in this region are shown by mutational analysis to play a role in the regulation of transcription. Gel shift analysis with oligonucleotides and nuclear extracts of ZR-75 showed protein binding to both of these sites. Spl binding activity is similar in ZR-75 and HT1080 cells whereas binding of factors to the E-MUC1 oligonucleotide reveals quantitative and qualitative differences between epithelial and non-epithelial cells.

Thus, the promoter of the MUC1 gene is useful for expressing cytokines, according to the method of the invention, in pancreatic and breast tumours.

<u>Example 9: Treatment of patients</u>

1. **Patient selection**

a) Patients with metastatic malignant melanoma with good performance data (WHO Grade zero 1 or 2) with a life expectancy of at least three months, normal renal and liver function and haematology, normal bilirubin and no evidence of cerebral secondaries are selected.

b) Written consent is obtained.

c) Patients need not have received prior chemotherapy because of the low activity, toxicity and immunosuppression of such treatments. They can be administered after the gene therapy is completed, if indicated.

d) Diagnosis of metastasis is confirmed by fine needle aspiration

cytology.

2. Administration of constructs

a) The constructs used are composed of a 769 bp fragment or a 2.5 kb fragment of the 5′ flanking sequence of the murine tyrosinase gene driving the human IL-2 gene within the promoterless mammalian expression vector pNASSβ (Clontech, Ca, USA). The decision to use the murine promoter sequence is based upon our demonstration that this sequence works well in human cells. Initial purification of the bulk grown plasmids DNA is achieved using QIAGEN-tips for plasmid purification (this is an anion exchange resin). The bacterial cells used as recipients for the plasmid constructs are the *E. coli* strain JM109. Verification of plasmid purity is by agarose gel electrophoresis. It is prepared to the same pyrogen free standards as monoclonal antibodies which are given in much higher amounts. It is administered in sterile saline.

b) All injections are given by a qualified medical practitioner with MRCP or equivalent and training in medical oncology. A 27 gauge needle is used and local anaesthetic administered first.

c) Patients are admitted for 24 hours following the injection and will be seen at three days and one week and thereafter weekly for one month and then monthly. The injection site is carefully examined and analgesia given as necessary.

3. Studies on initial needle aspirate for diagnostic purposes

a) immunocytochemistry for melanoma cells and assessment of cell cycle distribution.

b) PCR to assess cytokine expression - IL-2, interferon-γ and TNFα.

4. **Dosage schedule**

tyrosinase/IL-2

	Dose	Biopsy
Cohort 1	100 μg DNA/200 μl	1 week
2	100 μg DNA/200 μl	2 weeks

5. **Studies of excisional biopsy after construct injection**

a) immunochemistry for melanoma cells.

b) genomic PCR to assess the construct.

c) staining for lymphocyte sub-populations and dendritic cells, PCR for IL-2 interferon-γ and TNFα. *In situ* hybridisation for the same cytokines.

d) assessment of cytotoxic T cell response to autologous melanoma cells. Cells obtained from the biopsies will be used in chromium release assays, as well as peripheral T cells.

6. **Studies on stored DNA preparations**

a) In order to verify that the prepared DNA has not been degraded, routine examination of an aliquot of the injected material by agarose gel electrophoresis should be carried out.

<u>Assessment of results</u>

The effectiveness of this approach is assessed by three criteria.

<u>1) Assessment of IL-2 expression by RTPCR *in situ* hybridisation and immunochemistry</u>

A similar level of expression within 10-15% of tumour cells is found.

2) Assessment of local immune response by immunocytochemistry

Lymphocyte subpopulations and dendritic cells are stained to assess subtypes of cells present after the injections.

3) Assessment of cytotoxic T cell responses

There is 1-2 weeks of local IL-2 production.

There is a demonstration of a positive T cell response.

Genes that can be expressed include cytokines such as TNFα, GM-CSF, IL-4, interferon-γ or the proteins involved in T cell antigen recognition like class 1 molecules or B7.

Safety

Considering the life expectancy of these patients who already have metastatic cancer, the risks of insertion of genetic material into the somatic cells of the body would appear to be minimal. Clearly there may be events resulting from positional integration into the genome, eg insertional mutagenesis, inactivation or enhancement of expression, which could theoretically be deleterious. However, these have not manifested themselves in over 200 injections into recipient mice and their importance appears to be more theoretical than practical. Moreover, should adverse immunological reactions occur, they are unlikely to be beyond control with a range of immunosuppressive agents. Again, the short life expectancy of these patients makes long term undesirable sequelae an unlikely event. The risks of chemotherapy with marrow suppression, allergic reactions, Budd-Chiari syndrome and infection would all seem to

pose much greater clinical problems than the local injection of DNA.

Example 10: Co-injection of IL-2 expressing and B7-expressing DNA constructs into a melanoma

A TRP-1-B7 construct is made using PCR, the sequence information in the sequence listing and a DNA vector such that expression of the B7 coding sequence is driven by the TRP-1 promoter.

The TRP-1-B7 construct and the TRP-1-IL-2 construct of Example 3 are prepared in sterile, pyrogen free water. 100 μg of each DNA construct in 200 μl of water is injected into the melanoma at weekly intervals until the tumour regresses.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Imperial Cancer Research Technology Ltd
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(I) TELEX: 265107 ICRF G

(ii) TITLE OF INVENTION: Tumour therapy

(iii) NUMBER OF SEQUENCES: 22

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3281 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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GAGCTCCTCA CACGGACTCT GTCAGCTCCT CCCTGCAGCC TATCGGCCGC CCACCTGAGG     60
CTTGTCGGCC GCCCACTTGA GGCCTGTCGG CTGCCCTCTG CAGGCAGCTC CTGTCCCCTA    120
CACCCCCTCC TTCCCCGGGC TCAGCTGAAA GGGCGTCTCC CAGGGCAGCT CCCTGTGATC    180
TCCAGGACAG CTCAGTCTCT CACAGGCTCC GACGCCCCCT ATGCTGTCAC CTCACAGCCC    240
TGTCATTACC ATTAACTCCT CAGTCCCATG AAGTTCACTG AGCGCCTGTC TCCCGGTTAC    300
AGGAAAACTC TGTGACAGGG ACCACGTCTG TCCTGCTCTC TGTGGAATCC CAGGGCCCAG    360
CCAGTGCCTG ACACGGAACA GATGCTCCAT AAATACTGGT TAAATGTGTG GGAGATCTCT    420
AAAAAGAAAC ATATCACCTC CGTGTGGCCC CCAGCAGTCA GAGTCTGTTC CATGTGGACA    480
CAGGGGCACT GGCACCAGCA TGGGAGGAGG CCAGCAAGTG CCCGCGGCTG CCCCAGGAAT    540
GAGGCCTCAA CCCCCAGAGC TTCAGAAGGG AGGACAGAGG CCTGCAGGGA ATAGATCCTC    600
CGGCCTGACC CTGCAGCCTA ATCCTGAGTT CAGGGTCAGC TCACACCACG TCGACCCTGG    660
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TCAGCATCCC TAGGGCAGTT CCAGACAAGG CCGGAGGTCT CCTCTTGCCC TCCAGGGGGT 720

GACATTGCAC ACAGACATCA CTCAGGAAAC GGATTCCCCT GGACAGGAAC CTGGCTTTGC 780

TAAGGAAGTG GAGGTGGAGC CTGGTTTCCA TCCCTTGCTC CAACAGACCC TTCTGATCTC 840

TCCCACATAC CTGCTCTGTT CCTTTCTGGG TCCTCTGAGG ACCTGTTCTG CCAGGGGTCC 900

CTGTGCAACT CCAGACTCCC TCCTGGTACC ACCATGGGGA AGGTGGGGTG ATCACAGGAC 960

AGTCAGCCTC GCAGAGACAG AGACCACCCA GGACTGTCAG GGAGAACATG GACAGGCCCT 1020

GAGCCGCAGC TCAGCCAACA GACACGGAGA GGGAGGGTCC CCCTGGAGCC TTCCCCAAGG 1080

ACAGCAGAGC CCAGAGTCAC CCACCTCCCT CCACCACAGT CCTCTCTTTC CAGGACACAC 1140

AAGACACCTC CCCCTCCACA TGCAGGATCT GGGGACTCCT GAGACCTCTG GGCCTGGGTC 1200

TCCATCCCTG GGTCAGTGGC GGGGTTGGTG GTACTGGAGA CAGAGGGCTG GTCCCTCCCC 1260

AGCCACCACC CAGTGAGCCT TTTTCTAGCC CCCAGAGCCA CCTCTGTCAC CTTCCTGTTG 1320

GGCATCATCC CACCTTCCCA GAGCCCTGGA GAGCATGGGG AGACCCGGGA CCTGCTGGGT 1380

TTCTCTGTCA CAAAGGAAAA TAATCCCCCT GGTGTGACAG ACCCAAGGAC AGAACACAGC 1440

AGAGGTCAGC ACTGGGGAAA GACAGGTTGT CCACAGGGGA TGGGGGTCCA TCCACCTTGC 1500

CGAAAAGATT TGTCTGAGGA ACTGAAAATA GAAGGGAAAA AAGAGGAGGG ACAAAAGAGG 1560

CAGAAATGAG AGGGGAGGGG ACAGAGGACA CCTGAATAAA GACCACACCC ATGACCCACG 1620

TGATGCTGAG AAGTACTCCT GCCCTAGGAA GAGACTCAGG GCAGAGGGAG GAAGGACAGC 1680

AGACCAGACA GTCACAGCAG CCTTGACAAA ACGTTCCTGG AACTCAAGCT CTTCTCCACA 1740

GAGGAGGACA GAGCAGACAG CAGAGACCAT GGAGTCTCCC TCGGCCCCTC CCCACAGATG 1800

GTGCATCCCC TGGCAGAGGC TCCTGCTCAC AGGTGAAGGG AGGACAACCC CTGGGAGAGG 1860

GTGGGAGGAG GGAGCACAGA GACTGGCTGG GGTCTCCTGG GTAGGACAGG GCTGTGAGAC 1920

GGACAGAGGG CTCCTGTTGG AGCCTGAATA GGGAAGAGGA CATCAGAGAG GGACAGGAGT 1980

CACACCAGAA AAATCAAATT GAACTGGAAT TGGAAAGGGG CAGGAAAACC TCAAGAGTTC 2040

TATTTTCCTA GTTAATTGTC ACTGGCCACT ACGTTTTTAA AAATCATAAT AACTGCATCA 2100

GATGACACTT TAAATAAAAA CATAACCAGG GCATGAAACA CTGTCCTCAT CCGCCTACCG 2160

CGGACATTGG AAAATAAGCC CCAGGCTGTG GAGGGCCCTG GGAACCCTCA TGAACTCATC 2220

CACAGGAATC TGCAGCCTGT CCCAGGCACT GGGTGCAACC AAGATCACAC AAATCCCTGC 2280

CCTCATGAAG CTCATGCTCT CATGGGGAGG AAGACAGACA TACAAAGAGA TCTAGAATGT 2340

GAGGTCAGGT GTTGACAAGA GCCTGGAGGG AATAGAGCAG GGAAAGGTCA GAAAAGGAAG 2400

ACCCAAGGTC TCTAGAGGAG GTGTCAGGGA AGGGATCTCC CAAGAATGCC CTGATGTGAG 2460

CAGGACCTGA AGGCAATGGG GAGGGAGCCG TGAAGACCCC TGGAAAAGCA GATTCCACAC 2520

AGGGAAATGC CAAGGTCGGA GGTGCTAAGG AAATAGGAGA CACACTGCTG ACCTTGACCT 2580

AGTAGGACAC ACACACACAC ACACACACAC ACTCACTCAC TCCAGGGCTG GGGGATGAAG 2640

AGACCTGCTC AGGACCCAGG ACCCCATTTT TCCACCCTAA TGCATAGGTC CCAATATTGA 2700

CCGATGCTCT CTGCTCTCTC CTAGCCTCAC TTCTAACCTT CTGGAACCCG CCCACCACTG 2760

CCAAGCTCAC TATTGAATCC ACGCCGTTCA ATGTCGCAGA GGGGAAGGAG GTGCTTCTAC 2820

TTGTCCACAA TCTGCCCCAG CATCTTTTTG GCTACAGCTG GTACAAAGGT GAAAGAGTGG 2880

ATGGCAACCG TCAAATTATA GGATATGTAA TAGGAACTCA ACAAGCTACC CCAGGGCCCG 2940

CATACAGTGG TCGAGAGATA ATATACCCCA ATGCATCCCT GCTGATCCAG AACATCATCC 3000

AGAATGACAC AGGATTCTAC ACCCTACACG TCATAAAGTC AGATCTTGTG AATGAAGAAG 3060

CAACTGGCCA GTTCCGGGTA TACCGTGAGT GATTCCCCCA TGACCTCTGG GTGTTGGGGG 3120

TCAGTTCTAC TTCCCACACA CAGGATTATC AGGCCTGGGC TGTGCTGTGG CCCCCTCTGC 3180

ATTACGAACC ATGTTAGGGT TTGGGCATTT AGTGCAGGAT ACACACAGAA GAGACAAACT 3240

TCAACAGATC AGAATTCCTT TCCGGCATCC AGACCCTGCA G 3281

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7130 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GAATTCCACA TTGTTTGCTG CACGTTGGAT TTTGAAATGC TAGGGAACTT TGGGAGACTC 60

ATATTTCTGG GCTAGAGGAT CTGTGGACCA CAAGATCTTT TTATGATGAC AGTAGCAATG 120

TATCTGTGGA GCTGGATTCT GGGTTGGGAG TGCAAGGAAA AGAATGTACT AAATGCCAAG 180

ACATCTATTT CAGGAGCATG AGGAATAAAA GTTCTAGTTT CTGGTCTCAG AGTGGTGCAG 240

GGATCAGGGA GTCTCACAAT CTCCTGAGTG CTGGTGTCTT AGGGCACACT GGGTCTTGGA 300

GTGCAAAGGA TCTAGGCACG TGAGGCTTTG TATGAAGAAT CGGGGATCGT ACCCACCCCC 360

TGTTTCTGTT TCATCCTGGG CATGTCTCCT CTGCCTTTGT CCCCTAGATG AAGTCTCCAT 420

GAGCTACAAG GGCCTGGTGC ATCCAGGGTG ATCTAGTAAT TGCAGAACAG CAAGTGCTAG 480

CTCTCCCTCC CCTTCCACAG CTCTGGGTGT GGGAGGGGGT TGTCCAGCCT CCAGCAGCAT 540

GGGGAGGGCC TTGGTCAGCC TCTGGGTGCC AGCAGGGCAG GGGCGGAGTC CTGGGGAATG 600

AAGGTTTTAT AGGGCTCCTG GGGGAGGCTC CCCAGCCCCA AGCTTACCAC CTGCACCCGG 660

AGAGCTGTGT CACCATGTGG GTCCCGGTTG TCTTCCTCAC CCTGTCCGTG ACGTGGATTG 720

GTGAGAGGGG CCATGGTTGG GGGGATGCAG GAGAGGGAGC CAGCCCTGAC TGTCAAGCTG 780

AGGCTCTTTC CCCCCCAACC CAGCACCCCA GCCCAGACAG GGAGCTGGGC TCTTTTCTGT 840

CTCTCCCAGC CCCACTTCAA GCCCATACCC CCAGCCCCTC CATATTGCAA CAGTCCTCAC 900

TCCCACACCA GGTCCCCGCT CCCTCCCACT TACCCCAGAA CTTTCTCCCC ATTGCCCAGC 960

CAGCTCCCTG CTCCCAGCTG CTTTACTAAA GGGGAAGTTC CTGGGCATCT CCGTGTTTCT 1020

CTTTGTGGGG CTCAAAACCT CCAAGGACCT CTCTCAATGC CATTGGTTCC TTGGACCGTA 1080

TCACTGGTCC ATCTCCTGAG CCCCTCAATC CTATCACAGT CTACTGACTT TTCCCATTCA 1140

GCTGTGAGTG TCCAACCCTA TCCCAGAGAC CTTGATGCTT GGCCTCCCAA TCTTGCCCTA 1200

GGATACCCAG ATGCCAACCA GACACCTCCT TCTTCCTAGC CAGGCTATCT GGCCTGAGAC 1260

AACAAATGGG TCCCTCAGTC TGGCAATGGG ACTCTGAGAA CTCCTCATTC CCTGACTCTT 1320

AGCCCCAGAC TCTTCATTCA GTGGCCCACA TTTTCCTTAG GAAAAACATG AGCATCCCCA 1380

GCCACAACTG CCAGCTCTCT GATTCCCCAA ATCTGCATCC TTTTCAAAAC CTAAAAACAA 1440

AAAGAAAAAC AAATAAAACA AAACCAACTC AGACCAGAAC TGTTTTCTCA ACCTGGGACT 1500

TCCTAAACTT TCCAAAACCT TCCTCTTCCA GCAACTGAAC CTGGCCATAA GGCACTTATC 1560

CCTGGTTCCT AGCACCCCTT ATCCCCTCAG AATCCACAAC TTGTACCAAG TTTCCCTTCT 1620

CCCAGTCCAA GACCCCAAAT CACCACAAAG GACCCAATCC CCAGACTCAA GATATGGTCT 1680

GGGCGCTGTC TTGTGTCTCC TACCCTGATC CCTGGGTTCA ACTCTGCTCC CAGAGCATGA 1740

AGCCTCTCCA CCAGCACCAG CCACCAACCT GCAAACCTAG GGAAGATTGA CAGAATTCCC 1800

AGCCTTTCCC AGCTCCCCCT GCCCATGTCC CAGGACTCCC AGCCTTGGTT CTCTGCCCCC 1860

GTGTCTTTTC AAACCCACAT CCTAAATCCA TCTCCTATCC GAGTCCCCCA GTTCCCCCTG 1920

TCAACCCTGA TTCCCCTGAT CTAGCACCCC CTCTGCAGGC GCTGCGCCCC TCATCCTGTC 1980

TCGGATTGTG GGAGGCTGGG AGTGCGAGAA GCATTCCCAA CCCTGGCAGG TGCTTGTGGC 2040

CTCTCGTGGC AGGGCAGTCT GCGGCGGTGT TCTGGTGCAC CCCCAGTGGG TCCTCACAGC 2100

TGCCCACTGC ATCAGGAAGT GAGTAGGGGC CTGGGGTCTG GGGAGCAGGT GTCTGTGTCC 2160

CAGAGGAATA ACAGCTGGGC ATTTTCCCCA GGATAACCTC TAAGGCCAGC CTTGGGACTG 2220

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CATTATGTCT CTTGGTAACT GGCTTCGGTT GTGTCTCTCC GTGTGACTAT TTTGTTCTCT 2400

CTCTCCCTCT CTTCTCTGTC TTCAGTCTCC ATATCTCCCC CTCTCTCTGT CCTTCTCTGG 2460

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GGAGGCCAAG GCAGGTAGAT CACCTGAGGT CAGGAGTTCG AGACCAGCCT GGCCAACTGG 2700

TGAAACCCCA TCTCTACTAA AAATACAAAA AATTAGCCAG GCGTGGTGGC GCATGCCTGT 2760

AGTCCCAGCT ACTCAGGAGG CTGAGGGAGG AGAATTGCTT GAACCTGGGA GGTTGAGGTT 2820
GCAGTGAGCC GAGACCGTGC CACTGCACTC CAGCCTGGGT GACAGAGTGA GACTCCGCCT 2880
CAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AGAAAAGAAA AGAAAAGAAA AGGAATCTTT 2940
TATCCCTGAT GTGTGTGGGT ATGAGGGTAT GAGAGGGCCC CTCTCACTCC ATTCCTTCTC 3000
CAGGACATCC CTCCACTCTT GGGAGACACA GAGAAGGGCT GGTTCCAGCT GGAGCTGGGA 3060
GGGGCAATTG AGGGAGGAGG AAGGAGAAGG GGGAAGGAAA ACAGGGTATG GGGGAAAGGA 3120
CCCTGGGGAG CGAAGTGGAG GATACAACCT TGGGCCTGCA GGCCAGGCTA CCTACCCACT 3180
TGGAAACCCA CGCCAAAGCC GCATCTACAG CTGAGCCACT CTGAGGCCTC CCCTCCCCGG 3240
CGGTCCCCAC TCAGCTCCAA AGTCTCTCTC CCTTTTCTCT CCCACACTTT ATCATCCCCC 3300
GGATTCCTCT CTACTTGGTT CTCATTCTTC CTTTGACTTC CTGCTTCCCT TTCTCATTCA 3360
TCTGTTTCTC ACTTTCTGCC TGGTTTTGTT CTTCTCTCTC TCTTTCTCTG GCCCATGTCT 3420
GTTTCTCTAT GTTTCTGTCT TTTCTTTCTC ATCCTGTGTA TTTTCGGCTC ACCTTGTTTG 3480
TCACTGTTCT CCCCTCTGCC CTTTCATTCT CTCTGTCCTT TTACCCTCTT CCTTTTTCCC 3540
TTGGTTTCTC TCAGTTTCTG TATCTGCCCT TCACCCTCTC ACACTGCTGT TTCCCAACTC 3600
GTTGTCTGTA TTTTTGGCCT GAACTGTGTC TTCCCCAACC CTGTGTTTTT CTCACTGTTT 3660
CTTTTTCTCT TTTGGAGCCT CCTCCTTGCT CCTCTGTCCC TTCTCTCTTT CCTTATCATC 3720
CTCGCTCCTC ATTCCTGCGT CTGCTTCCTC CCCAGCAAAA GCGTGATCTT GCTGGGTCGG 3780
CACAGCCTGT TTCATCCTGA AGACACAGGC CAGGTATTTC AGGTCAGCCA CAGCTTCCCA 3840
CACCCGCTCT ACGATATGAG CCTCCTGAAG AATCGATTCC TCAGGCCAGG TGATGACTCC 3900
AGCCACGACC TCATGCTGCT CCGCCTGTCA GAGCCTGCCG AGCTCACGGA TGCTGTGAAG 3960
GTCATGGACC TGCCCACCCA GGAGCCAGCA CTGGGGACCA CCTGCTACGC CTCAGGCTGG 4020
GGCAGCATTG AACCAGAGGA GTGTACGCCT GGGCCAGATG GTGCAGCCGG GAGCCCAGAT 4080
GCCTGGGTCT GAGGGAGGAG GGGACAGGAC TCCTGGGTCT GAGGGAGGAG GGCCAAGGAA 4140
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AACTTCAGTG TGTGGACCTC CATGTTATTT CCAATGACGT GTGTGCGCAA GTTCACCCTC 4260
AGAAGGTGAC CAAGTTCATG CTGTGTGCTG GACGCTGGAC AGGGGGCAAA AGCACCTGCT 4320
CGGTGAGTCA TCCCTACTCC CAAGATCTTG AGGGGAAAGG TGAGTGGGGA CCTTAATTCT 4380
GGGCTGGGGT CTAGAAGCCA ACAAGGCGTC TGCCTCCCCT GCTCCCCAGC TGTAGCCATG 4440
CCACCTCCCC GTGTCTCATC TCATTCCCTC CTTCCCTCTT CTTTGACTCC CTCAAGGCAA 4500
TAGGTTATTC TTACAGCACA ACTCATCTGT TCCTGCGTTC AGCACACGGT TACTAGGCAC 4560
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GCCCCTCCCT TCTGTAGCCC CCAAGCCAGT GAGGGGCACA GGCAGGAACA GGGACCACAA 4680
CACAGAAAAG CTGGAGGGTG TCAGGAGGTG ATCAGGCTCT CGGGGAGGGA GAAGGGGTGG 4740

GGAGTGTGAC	TGGGAGGAGA	CATCCTGCAG	AAGGTGGGAG	TGAGCAAACA	CCTGCCGCAG	4800
GGGAGGGGAG	GGCCCTGCGG	CACCTGGGGG	AGCAGAGGGA	ACAGCATCTG	GCCAGGCCTG	4860
GGAGGAGGGG	CCTAGAGGGC	GTCAGGAGCA	GAGAGGAGGT	TGCCTGGCTG	GAGTGAAGGA	4920
TCGGGGCAGG	GTGCGAGAGG	GAAGAAAGGA	CCCCTCCTGC	AGGGCCTCAC	CTGGGCCACA	4980
GGAGGACACT	GCTTTTCCTC	TGAGGAGTCA	GGAACTGTGG	ATGGTGCTGG	ACAGAAGCAG	5040
GACAGGGCCT	GGCTCAGGTG	TCCAGAGGCT	GCCGCTGGCC	TCCCTATGGG	ATCAGACTGC	5100
AGGGAGGGAG	GGCAGCAGGG	ATGTGGAGGG	AGTGATGATG	GGGCTGACCT	GGGGGTGGCT	5160
CCAGGCATTG	TCCCCACCTG	GGCCCTTACC	CAGCCTCCCT	CACAGGCTCC	TGGCCCTCAG	5220
TCTCTCCCCT	CCACTCCATT	CTCCACCTAC	CCACAGTGGG	TCATTCTGAT	CACCGAACTG	5280
ACCATGCCAG	CCCTGCCGAT	GGTCCTCCAT	GGCTCCCTAG	TGCCCTGGAG	AGGAGGTGTC	5340
TAGTCAGAGA	GTAGTCCTGG	AAGGTGGCCT	CTGTGAGGAG	CCACGGGGAC	AGCATCCTGC	5400
AGATGGTCCT	GGCCCTTGTC	CCACCGACCT	GTCTACAAGG	ACTGTCCTCG	TGGACCCTCC	5460
CCTCTGCACA	GGAGCTGGAC	CCTGAAGTCC	CTTCCCTACC	GGCCAGGACT	GGAGCCCCTA	5520
CCCCTCTGTT	GGAATCCCTG	CCCACCTTCT	TCTGGAAGTC	GGCTCTGGAG	ACATTTCTCT	5580
CTTCTTCCAA	AGCTGGGAAC	TGCTATCTGT	TATCTGCCTG	TCCAGGTCTG	AAAGATAGGA	5640
TTGCCCAGGC	AGAAACTGGG	ACTGACCTAT	CTCACTCTCT	CCCTGCTTTT	ACCCTTAGGG	5700
TGATTCTGGG	GGCCCACTTG	TCTGTAATGG	TGTGCTTCAA	GGTATCACGT	CATGGGGCAG	5760
TGAACCATGT	GCCCTGCCCG	AAAGGCCTTC	CCTGTACACC	AAGGTGGTGC	ATTACCGGAA	5820
GTGGATCAAG	GACACCATCG	TGGCCAACCC	CTGAGCACCC	CTATCAACTC	CCTATTGTAG	5880
TAAACTTGGA	ACCTTGGAAA	TGACCAGGCC	AAGACTCAAG	CCTCCCCAGT	TCTACTGACC	5940
TTTGTCCTTA	GGTGTGAGGT	CCAGGGTTGC	TAGGAAAAGA	AATCAGCAGA	CACAGGTGTA	6000
GACCAGAGTG	TTTCTTAAAT	GGTGTAATTT	TGTCCTCTCT	GTGTCCTGGG	GAATACTGGC	6060
CATGCCTGGA	GACATATCAC	TCAATTTCTC	TGAGGACACA	GATAGGATGG	GGTGTCTGTG	6120
TTATTTGTGG	GATACAGAGA	TGAAAGAGGG	GTGGGATCCA	CACTGAGAGA	GTGGAGAGTG	6180
ACATGTGCTG	GACACTGTCC	ATGAAGCACT	GAGCAGAAGC	TGGAGGCACA	ACGCACCAGA	6240
CACTCACAGC	AAGGATGGAG	CTGAAAACAT	AACCCACTCT	GTCCTGGAGG	CACTGGGAAG	6300
CCTAGAGAAG	GCTGTGAGCC	AAGGAGGGAG	GGTCTTCCTT	TGGCATGGGA	TGGGGATGAA	6360
GTAAGGAGAG	GGACTGGACC	CCCTGGAAGC	TGATTCACTA	TGGGGGGAGG	TGTATTGAAG	6420
TCCTCCAGAC	AACCCTCAGA	TTTGATGATT	TCCTAGTAGA	ACTCACAGAA	ATAAAGAGCT	6480
CTTATACTGT	GGTTTATTCT	GGTTTGTTAC	ATTGACAGGA	GACACACTGA	AATCAGCAAA	6540
GGAAACAGGC	ATCTAAGTGG	GGATGTGAAG	AAAACAGGGA	AAATCTTTCA	GTTGTTTTCT	6600
CCCAGTGGGG	TGTTGTGGAC	AGCACTTAAA	TCACACAGAA	GTGATGTGTG	ACCTTGTGTA	6660
TGAAGTATTT	CCAACTAAGG	AAGCTCACCT	GAGCCTTAGT	GTCCAGAGTT	CTTATTGGGG	6720

GTCTGTAGGA TAGGCATGGG GTACTGGAAT AGCTGACCTT AACTTCTCAG ACCTGAGGTT 6780

CCCAAGAGTT CAAGCAGATA CAGCATGGCC TAGAGCCTCA GATGTACAAA AACAGGCATT 6840

CATCATGAAT CGCACTGTTA GCATGAATCA TCTGGCACGG CCCAAGGCCC CAGGTATACC 6900

AAGGCACTTG GGCCGAATGT TCCAAGGGAT TAAATGTCAT CTCCCAGGAG TTATTCAAGG 6960

GTGAGCCCTG TACTTGGAAC GTTCAGGCTT TGAGCAGTGC AGGGCTGCTG AGTCAACCTT 7020

TTACTGTACA GGGGGGTGAG GGAAAGGGAG AAGATGAGGA AACCGCCTAG GGATCTGGTT 7080

CTGTCTTGTG GCCGAGTGGA CCATGGGGCT ATCCCAAGAA GGAGGAATTC 7130

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 858 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CGAGCGGCCC CTCAGCTTGC GCGGCCCAGC CCCGCAAGGC TCCCGGTGAC CACTAGAGGG 60

CGGGAGGAGC TCCTGGCCAG TGGTGGAGAG TGGCAAGGAA GGACCCTAGG GTTCATCGGA 120

GCCCAGGTTT ACTCCCTTAA GTGGAAATTT CTTCCCCCAC TCCTCCTTGG CTTTCTCCAA 180

GGAGGGAACC CAGGCTGCTG GAAAGTCCGG CTGGGGGGGG GACTGTGGGT TCAGGGGAGA 240

ACGGGGTGTG GAACGGGACA GGGAGCGGTT AGAAGGGTGG GGCTATTCCG GGAAGTGGTG 300

GGGGGAGGGA GCCCAAAACT AGCACCTAGT CCACTCATTA TCCAGCCCTC TTATTTCTCG 360

GCCGCTCTGC TTCAGTGGAC CCGGGGAGGG CGGGGAAGTG GAGTGGGAGA CCTAGGGGTG 420

GGCTTCCCGA CCTTGCTGTA CAGGACCTCG ACCTAGCTGG CTTTGTTCCC CATCCCCACG 480

TTAGTTGTTG CCCTGAGGCT AAAACTAGAG CCCAGGGGCC CCAAGTTCCA GACTGCCCCT 540

CCCCCCTCCC CCGGAGCCAG GGAGTGGTTG GTGAAAGGGG GAGGCCAGCT GGAGAACAAA 600

CGGGTAGTCA GGGGGTTGAG GATTAGAGCC CTTGTACCCT ACCCAGGAAT GGTTGGGGAG 660

GAGGAGGAAG AGGTAGGAGG TAGGGGAGGG GGCGGGGTTT TGTCACCTGT CACCTGCTCG 720

CTGTGCCTAG GGCGGGCGGG CGGGGAGTGG GGGGACCGGT ATAAAGCGGT AGGCGCCTGT 780

GCCCGCTCCA CCTCTCAAGC AGCCAGCGCC TGCCTGAATC TGTTCTGCCC CCTCCCCACC 840

CATTTCACCA CCACCATG 858

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1581 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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CATGGTGTCC GACTTATGCC CGAGAAGATG TTGAGCAAAC TTATCGCTTA TCTGCTTCTC     60
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CAGGTCAACG GATCCCTTCT TGACCAGTAT AGCTGCATTC TTGGCTGGGG CATTCCAACT    180
AGAACTGCCA AATTTAGCAC ATAAAAATAA GGAGGCCCAG TTAAATTTGA ATTTCAGATA    240
AACAATGAAT AATTTGTTAG TATAAATATG TCCCATGCAA TATCTTGTTG AAATTAAAAA    300
AAAAAGTCTT CCTTCCATGC CCCACCCCTA CCACTAGGCC TAAGGAATAG GGTCAGGGGC    360
TCCAAATAGA ATGTGGTTGA GAAGTGGAAT TAAGCAGGCT AATAGAAGGC AAGGGGCAAA    420
GAAGAAACCT TGAATGCATT GGGTGCTGGG TGCCTCCTTA AATAAGCAAG AAGGGTGCAT    480
TTTGAAGAAT TGAGATAGAA GTCTTTTTGG GCTGGGTGCA GTTGCTCGTG GTTGTAATTC    540
CAGCACTTTG GGAGGCTGAG GCGGGAGGAT CACCTGAGGT TGGGAGTTCA AGACCAGCCT    600
CACCAACGTG GAGAACCCTG TCTTTACTAA AAATACAAAA AATTCAGCTG GTCATGGTGG    660
CACATGCCTG TAATCCCAGC TGCTCGGGAG GCTGAGGCAG GAGAATCACT TGAACCAGGG    720
AGGCAGAGGT TGTGGTGAGC AGAGATCGCG CCATTGCTCT CCAGCCTGGG CAACAAGAGC    780
AAAAGTTCGT TTAAAAAAAA AAAAAAGTCC TTTCGATGTG ACTGTCTCCT CCCAAATTTG    840
TAGACCCTCT TAAGATCATG CTTTTCAGAT ACTTCAAAGA TTCCAGAAGA TATGCCCCGG    900
GGGTCCTGGA AGCCACAAGG TAAACACAAC ACATCCCCCT CCTTGACTAT CAATTTTACT    960
AGAGGATGTG GTGGGAAAAC CATTATTTGA TATTAAAACA AATAGGCTTG GGATGGAGTA   1020
GGATGCAAGC TCCCCAGGAA AGTTTAAGAT AAAACCTGAG ACTTAAAAGG GTGTTAAGAG   1080
TGGCAGCCTA GGGAATTTAT CCCGGACTCC GGGGGAGGGG GCAGAGTCAC CAGCCTCTGC   1140
ATTTAGGGAT TCTCCGAGGA AAAGTGTGAG AACGGCTGCA GGCAACCCAG GCGTCCCGGC   1200
GCTAGGAGGG ACGACCCAGG CCTGCGCGAA GAGAGGGAGA AAGTGAAGCT GGGAGTTGCC   1260
GACTCCCAGA CTTCGTTGGA ATGCAGTTGG AGGGGGCGAG CTGGGAGCGC GCTTGCTCCC   1320
AATCACAGGA GAAGGAGGAG GTGGAGGAGG AGGGCTGCTT GAGGAAGTAT AAGAATGAAG   1380
TTGTGAAGCT GAGATTCCCC TCCATTGGGA CCGGAGAAAC CAGGGGAGCC CCCCGGGCAG   1440
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CCGCGCGCCC CTTCCCACGG GGCCCTTTAC TGCGCCGCGC GCCCGGCCCC CACCCCTCGC 1500

AGCACCCCGC GCCCCGCGCC CTCCCAGCCG GGTCCAGCCG GAGCCATGGG GCCGGAGCCG 1560

CAGTGAGCAC CATGGAGCTG G 1581

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1305 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1204..1284

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GGATCCGTCC CGGGACTAGC AGGGCTTTGG GCAGCAACCC GCAGGAGCCC GACCGCCTCT 60

GGCCAGGTCC GGGCAGCTGG TGGGGGAGGT TCCAGAGGTC CACGCCATTC GTGGACGCAG 120

TCTCTAGTGT CCTCTCCGCG TCCCACTTCA CTGCCCCATC CCCTTTCCTG CGAGAGCCTG 180

GACTTGGAAG GCACCTGGGA GGGTGTAAGC GCCTTGGTGT GTGCCCATCT GGGTCCCCAG 240

AAGAGCGGCG GGAACTGCGG CCGCCCGGAC GGTGCGGCCA GACTCCAGTG TGGAAGGGGA 300

GGCAGCTGTT CTCCCAGGCG GCCGTGGGGG GCAGCAGAGG GGACGGCGAC AGGTGCGGGA 360

GCCCCTCCCG GGGTAGAAGT GGAAAGGCGG GCTCCGGGGT CTGTTCCCAG GCTGGAAACC 420

ACCCCCGCCC CCCATCCAAA TCCCCGGGAG AGGCCCGGCC GGCGCCGGGT CTGGAGGAGG 480

AAGCGGCCAG AGACAGTGCA ATTTCACGCG GTCTCTGTGG CTCGGGTTCC TGGGCTGGGT 540

GGATGAATTA TGGGGTTTCG AGTCTGGGAG AAACTGAGGT GGCCTGGACG TGAGGCAAAA 600

AACACCCTCC CCCTCAAAAA CACACAGAGA GAAATATTCA CATTCTGAGA GAAAATCCAC 660

CAAGTGAACC AACCGGCTAG GGGAGTTGAG TGATTTGGTT AATGGGCGAG GCCAACTTTC 720

AGGGGGCAGG GCTTTGGAGA GCTTTCCACT CCCTCATTCA TTACCCTTCC CTGGATCTGG 780

GGGCTTTCGG AATCTCGACC TCCCCTTGGC CTATCTCCTG CAGAAAAATT AGGGTGAGCC 840

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GCGGTCCGAG GCTCCGCAAT CCCCACTCCA GCCTCGCGCG GGAGGGGGCG CGGCCCGTGT 960

GACTCACCCC CTTCCCTCTG CGTTCCTCCC TCCCTCTCTC TCTCTCTCTC ACACACACAC 1020

ACCCCTCCCC TGCCATCCCT CCCCGGACTC CGGCTCCGGC TCCGATTGCA ATTTGCAACC 1080

TCCGCTGCCG TCGCCGCAGC AGCCACCAAT TCGCCAGCGG TTCAGGTGGC TCTTGCCTCG 1140

ATGTCCTAGC CTAGGGGCCC CCGGGCCGGA CTTGGCTGGG CTCCCTTCAC CCTCTGCGGA 1200

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GTC ATG AGG GCG AAC GAC GCT CTG CAG GTG CTG GGC TTG CTT TTC AGC      1248
    Met Arg Ala Asn Asp Ala Leu Gln Val Leu Gly Leu Leu Phe Ser
     1               5                  10                  15

CTG GCC CGG GGC TCC GAG GTG GGC AAC TCT CAG GCA GGTAAGTGCC           1294
Leu Ala Arg Gly Ser Glu Val Gly Asn Ser Gln Ala
                20                  25

CAGAGAGCAC C                                                         1305
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(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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Met Arg Ala Asn Asp Ala Leu Gln Val Leu Gly Leu Leu Phe Ser Leu
 1               5                  10                  15

Ala Arg Gly Ser Glu Val Gly Asn Ser Gln Ala
            20                  25
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(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4752 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AATTCATGCC CCAGTTGACA ACATAGCTGG TTCAAGACAA ACCTGAGCAC TTTTCATCAC 60

TGAAATCTTC ACTCTGGACC AATCAACATT CATACATTCC CTTCTTTACT TTAACACTCT 120

CCTGAGAGCT ATTTCTCTTC TCATCCTAAT TCTCTGCTCA TATCACATTT CAGCAGCTTA 180

CATATGAAAA TCTTGTACAT TCCCATGAGA TTGCATTGAA ATTGCTTCAA CCCTTTTCTA 240

TGTCCATATG TATACCTTAC TTCTCATTCC TATTGCTTTG TAGCTACAGA AGCCTTAGCA 300

GTCTTTAGAA ATCTTGGGAG TGTTATGCCT CTTCCACTTA AAGCTTTAAT TCAAGAACTC 360

ATTCTCTAGA AGTTTAAACA ACTGCTATTC TGCTCTTCTA TGACTCTTTA ACATGTCTCT 420

CAAAATATGT TTTCTCCCAG AAAACTCTCC TCAACTTTCT TCAAAATTAA TAGACTACAT 480

TTTGCCCCAC AGAAATTCTT CTATAGGAAC TTCAATTTTT TTATTTAATC CAGGAAAACA 540
TATTTAAACA TACTTAGCTA TAAATATTAA GATTAAATTG TGATAAAAAC TTATTTTTGA 600
CAGTGGTGGA AACTGTCCTA TTAAATTTAC CATATCATTC CAATTTTATC CTCAGAAAAT 660
GCCCAATAGA TGATCAATAA ATATTTGCAG TATACTTATT GCATTCCCAG CTATAGCTAA 720
TATAGATTGC ATTAAGACTG TCTTAGGGTA GACAATAGTC AAACAGAATT GGATAGGGCA 780
TATTCAAATT CTGTAGCTAC CAAATGTGTA TACAAGGGAA AAATGTGTAA ATGAATACTT 840
ACATAGAAAG AGCAGGACTG GTGATAAGAT TGTCATGTAA ATAAGTTGGT GGAGACTTGA 900
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GAGCATGGGA AAGCCTTAGA AAATGAGCAT GGGAAAGCCC TTAGAAAGAG TGATGAGGAT 1080
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CACCCACACA CTGTTCTTTC TACCTCGCAA GGCTGCCTTG AATCTCAAGA GAATCCTATG 1440
GAGCAGTCAA CACATTTTAA ATACTGAAAC AAACCCTAGG GAAGAAAAGA AGCAGAGCAG 1500
GCTGACATTC CAGCATTATC AGGAAAGCAA TGATTTTCCT AGATTTCCGC AGCCCCAGTG 1560
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ACCACTCAGC TTTTAGTATG AAGCAGCATA GAGAAGGGAG TTCCAAAGAG ACGTTTGTTT 1680
CTTGACCATT CTCATTATCC TTCTTTCATG GAGCAGTGCT ATTCAAACCA TCCAGTAAGT 1740
CCATTACTCA CTTCCACATT TTATGAGCAA AATAATAAAA GAGAGATAGA GTAAGAATGA 1800
AGGAGAGAGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA GAGAGGAAGA 1860
GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGG 1920
TGTTTTCTTA ACTAGAAACT TTATGCATTG AAGCAGTTCA CCAAAATAAC AAAGTAACAA 1980
AGTAAGATAT CTTTGGAATA ATCAATTCAA GATAATCAAG GAAAAATGAG AGGCAACTAT 2040
TTTAGACTGA TTACTTTTAT AAAATAAATA AGCTCAGCTT AGCCAGATAT AAGCAATATT 2100
CTGAGTTCTG AAGAAAAATT TTTGACAAAA TGAGTTCTAT AAATGTTATT GTCTACTTAT 2160
GATCTCTAAA TACAACAGGC TTGTATTCAG AATCTAGATG TTTCATGACC TTTATTCATA 2220
AGAGATGATG TATTCTTGAT ACTACTTCTC ATTTGCAAAT TCCAATTATT ATTAATTTCA 2280
TATCAATTAG AATAATATAT CTTCCTTCAA TTTAGTTACC TCACTATGGG CTATGTACAA 2340
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TTTGTATGAT CTAAAGGAGA AAATGTTCTT GGCTGTTTTG TATTGCCTTC TGTGGAGTTT 2580
CCAGATCTCT GATGGCCATT TTCCTCGAGC CTGTGCCTCC TCTAAGAACT TGTTGGCAAA 2640
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AGGCACCTAT GGCCAAATGA ACAATGGGTC AACACCCATG TTTAATGATA TCAACATCTA 3060
CGACCTCTTT GTATGGATGC ATTACTATGT GTCAAGGGAC ACACTGCTTG GGGGCTCTGA 3120
AATATGGAGG GACATTGATT TTGCCCATGA AGCACCAGGG TTTCTGCCTT GGCACAGACT 3180
TTTCTTGTTA TTGTGGGAAC AAGAAATTCG AGAACTAACT GGGGATGAGA ACTTCACTGT 3240
TCCATACTGG GATTGGAGAG ATGCAGAAAA CTGTGACATT TGCACAGATG AGTACTTGGG 3300
AGGTCGTCAC CCTGAAAATC CTAACTTACT CAGCCCAGCA TCCTTCTTCT CCTCCTGGCA 3360
GGTAAGATGC ACTATATAGA GAGAGTTGCA AAGACTGGTA CTTCAGCAGC CACATTTTCA 3420
TGCTCTGTGA GCATCTCTGA TAATATCTCA GGGCAGAAAA TGTGCCTTAC TAACAGATGT 3480
TAATGCTTCT TGATTTCTTT TTCTCTTTTG AGAACTCTTC AAAGTGTTAT TAAACAAATA 3540
TCTATGTGCT TATTTGTCTT AATATCTAAC AGCTTAGTTA GATTTCTAAG CTGCTATAAA 3600
CAAGGACTGA TTGGTTCACC ACTGTATTGT TAGCACCTCC TATGTATCTA ATAACAGTAA 3660
CTCAGTTATT AAGAATGGAT AGAAACCAGA TTATCTTAGT TCAATTTCTA GTAATATTAA 3720
ACTTAATATA ACAGTAAATC CATAAGTATC TACTTAAAAT ATAATCTCTG GCCAAACCAA 3780
GACTTATTAT TAGGATCTTC AAGAGAAAGT GCTGAGATAA TTCACTAAGT ATCAGAGATG 3840
ACCTTTATTA CATGATTGCC TGATAGAAAA AATGATTACA CACACACAAA AAAATCTTCA 3900
GTTGCTTAAT TTAAGCGCTG ACTCTCAACA GTTAAGTAAT AAAAGAGTTA AGCCTGCTGT 3960
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TTCTATTTTA CATCATAAGA TACTACTTAA TTTGTTAAAA ATTATTTTTT ATACATTGTT 4080
GTAAATACAA AGTGATATTT CTAATGATTA CAAGGCTGTC TGGCTAACTT ACGTTATGTT 4140
CAGGAGAAGA CAGTCCTTTT TAAGGAATGG GCACTTTCTA ACTTTTTTTC TCTAGGATGG 4200
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TTTGAGATTA AATTTTCTTT TCTCCTATTA TTTTTCCTCA TTCTGGAATG GAAGCAAAAG 4320
ATGAAGAAAG AAATATATGT TAAATTGTTT TCCTTTAAAT GAACACAAAT GTGAAATATG 4380
TTTTTCTGCC TATCTTGTAA AATTTTCTAT TGCAACTATT CTGATTACCA GTTCAAATGG 4440

GGAAAAAAGA ACATAGGCTA CCCCACACTT GAAATTTTGA AATATGAATG TCCTCTGTCT 4500

CTAGCTGAGT ACTCTGGCGC TTCCAAAATG GAAACCTTTA AAGGGCCACT GTAAATTACA 4560

GCTGCTAATT CCTGGTGCCA ATGGTGATAA GTGTTTACTA AACCTAGTGA GTACTTTATA 4620

GCATGGGTCT GCTGCGAAGT AACATTGCTG TATATTTTCA GTCATTCTAC CTTAATTCAT 4680

GAACTGCAAA ACTCTCATCT AGCTTTTTAC TTCTCTAGCT ATTGCTTTAA GTTCTATCAG 4740

GCTCAGGTGT GG 4752

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1236 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AAGCTTTGTA GAGTAATCAT GTATTCCAAA CTCAGGCTTA CATTTGAATG TTGGCTACAT 60

ATGTATGAGT TTTCAACTTC CAGGAGAAAA CGTCTCTTTA AAAGAGAACA ACCAAAAGCT 120

AACAGAAAAT ACAAGTGTGA CATTGGCCTT AGTTCGACCA AGAAAGCAAT TCATCTTGTT 180

TCTTCCTTTG TGGTATACAG ATAAGAAAAA TAAAATCACT ACAACGAAAG CAAAATCTCT 240

TCAGCGTCTC TAATACATCT TCCAAATCAG TGTGTCTGAC CTTTTCTTAA GACTTTAACC 300

ATCACAAGGA AACCAGTGGG GAGGGAGTCA TGTGCTGCCT AGTAGTTAAA GGGCAGGAGA 360

ATTCACTGGT GTGAGAAGGG ATTAGTGAGA GCTGGAAGAG AGGACCAGCC CCTCCCAGTG 420

TGAGGAATCT GGCTTGGGAT TTACTGTCTG GCAGAAAATC TCTTCGGGCA ATTAACAGCT 480

GGCATCAGGG GAAAAGCAGA CATCCAACAA CACTAGCTCT GAAGGAGATC AGCAGAGAAA 540

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CCATGGTTAA CTATTACTAT GGTAGTCACC AACTAGTGGA TGCTTTTGGT AAATGACATC 720

TATGGAAAGT CTTTTTGGAT CAGGGTGATC TTTTTATGTA TGTGTATGTG CATGGATATG 780

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AAAGGTGAAT AATTATTTTG ACTATTGTTT AGAAATGTTG TTTCACATGA AAGATTCCAT 1080

TTCCGGAGTG GGTTGAAAAG TATGCAAAAG AACTTTTGCA ACTCTGTTTT TGCCTTTCTG 1140

TTTTTCAGCT GTATTTTCAT CTGAGCACCC CTGTCTTCTC CATGCAAAGA GCAGCATAGG 1200

AGACCTGTGT TCTGAACTCT TGCTTCGAGA AGAATG 1236

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5737 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TTAATCAACA AATCTAAACA TTTATTCTTT TCATCTGTTT ACTCTTGCTC TTGTTCACCA 60

CAATATGCTA TTCACATGTT CAGTGTAGTT TTATGACAAA GAAAATTTTC TGAGTTACTT 120

TTGTATCCCC ACCCCCTTAA AGAAAGGAGG AAAAACTGTT TCATACAGAA GGCGTTAATT 180

GCATGAATTA GAGCTATCAC CTAAGTGTGG GCTAATGTAA CAAAGAGGGA TTTCACCTAC 240

ATCCATTCAG TCAGTCTTTG GGGGTTTAAA GAATTCCAAA GAGTCATCAG AAGAGGAAAA 300

ATGAAGGTAA TGTTTTTTCA GACAGGTAAA GTCTTTGAAA ATATGTGTAA TATGTAAAAC 360

ATTTTGACAC CCCCATAATA TTTTTCCAGA ATTAACAGTA TAAATTGCAT CTCTTGTTCA 420

AGAGTTCCCT ATCACTCTCT TTAATCACTA CTCACAGTAA CCTCAACTCC TGCCACAATG 480

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CCTACTTCAA GTTCTACAAA GAAAACACAG CTACAACTGG AGCATTTACT GCTGGATTTA 600

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AAGAATCCCA AACTCACCAG GATGCTCACA TTTAAGTTTT ACATGCCCAA GAAGGTAAGT 780

ACAATATTTT ATGTTCAATT TCTGTTTTAA TAAAATTCAA AGTAATATGA AAATTTGCAC 840

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TAAAAATCTT TTCTTGTTTT AGGAAAGGTT TCTAAGTGAG AGGCAGCATA ACACTAATAG 1200

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AACCGTGTCT GGCATAAGGT AAATACCATA CAAGCATTAG CTATTATTAG TAGTATTAAA 1440
GATAAAATTT TCACTGAGAA ATACAAAGTA AAATTTTGGA CTTTATCTTT TTACCAATAG 1500
AACTTGAGAT TTATAATGCT ATATGACTTA TTTTCCAAGA TTAAAAGCTT CATTAGGTTG 1560
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CAATCCCATT GACTCAAATT CTATTAATTC AAGAGAAATC TGCTTCTAAC TACCCTTCTG 1800
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CATGCCAAAA ACTTAAACCC GAATTTGTAG AATTTGTAGT GGTGAATTGA AAGCGCAATA 1980
GATGGACATA TCAGGGGATT GGTATTGTCT TGACCTACCT TTCCCACTAA AGAGTGTTAG 2040
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AACCAAAAGT AATGATAAAC TCTATTCATT TGTTCATTTA ACCCTCATTG CACATTTACA 2160
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AGTGTGCTTA TTTATAAAGT GGAGATGATA CTTCACTGCC TACTTCAAAA GATGACTGTG 2340
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ACATTTTGCA AAGCCAAATT AAGCTAAAAC CAGTGAGTCA ACTATCACTT AACGCTAGTC 2880
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ATTAAAAAAA TACATTGAGA TCAATGTCAT CTAGACATTG ACAGATTCAG TTCCTTATCT 4020
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TTAAATTTTA TATTTATTGT TGAATGTATG GTTTGCTACC TATTGTAACT ATTATTCTTA 5280

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TTGAAACTCT TCATAGATTT GGTGTGGCTA TGAATTC 5737

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 614 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GATCGTTAGC TTCTCCTGAT AAACTAATTG CCTCACATTG TCACTGCAAA TCGACACCTA 60

TTAATGGGTC TCACCTCCCA ACTGCTTCCC CCTCTGTTCT TCCTGCTAGC ATGTGCCGGC 120

AACTTTGTCC ACGGACACAA GTGCGATATC ACCTTACAGG AGATCATCAA AACTTTGAAC 180

AGCCTCACAG AGCAGAAGAC TCTGTGCACC GAGTTGACCG TAACAGACAT CTTTGCTGCC 240

TCCAAGAACA CAACTGAGAA GGAAACCTTC TGCAGGGCTG CGACTGTGCT CCGGCAGTTC 300

TACAGCCACC ATGAGAAGGA CACTCGCTGC CTGGGTGCGA CTGCACAGCA GTTCCACAGG 360

CACAAGCAGC TGATCCGATT CCTGAAACGG CTCGACAGGA ACCTCTGGGG CCTGGCGGGC 420

TTGAATTCCT GTCCTGTGAA GGAAGCCAAC CAGAGTACGT TGGAAAACTT CTTGGAAAGG 480

CTAAAGACGA TCATGAGAGA GAAATATTCA AAGTGTTCGA GCTGAATATT TTAATTTATG 540

AGTTTTTGAT AGCTTTATTT TTTAAGTATT TATATATTTA TAACTCATCA TAAAATAAAG 600

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(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1589 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

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CTGAAAGTAC ACTGCTGGCG GATCCTACGG AAGTTATGGA AAAGGCAAAG CGCAGAGCCA     180
CGCCGTAGTG TGTGCCGCCC CCCTTGGGAT GGATGAAACT GCAGTCGCGG CGTGGGTAAG     240
AGGAACCAGC TGCAGAGATC ACCCTGCCCA ACACAGACTC GGCAACTCCG CGGAAGACCA     300
GGGTCCTGGG AGTGACTATG GGCGGTGAGA GCTTGCTCCT GCTCCAGTTG CGGTCATCAT     360
GACTACGCCC GCCTCCCGCA GACCATGTTC CATGTTTCTT TTAGGTATAT CTTTGGACTT     420
CCTCCCCTGA TCCTTGTTCT GTTGCCAGTA GCATCATCTG ATTGTGATAT TGAAGGTAAA     480
GATGGCAAAC AATATGAGAG TGTTCTAATG GTCAGCATCG ATCAATTATT GGACAGCATG     540
AAAGAAATTG GTAGCAATTG CCTGAATAAT GAATTTAACT TTTTTAAAAG ACATATCTGT     600
GATGCTAATA AGGAAGGTAT GTTTTTATTC CGTGCTGCTC GCAAGTTGAG GCAATTTCTT     660
AAAATGAATA GCACTGGTGA TTTTGATCTC CACTTATTAA AAGTTTCAGA AGGCACAACA     720
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CAACCAACAA AGAGTTTGGA AGAAAATAAA TCTTTAAAGG AACAGAAAAA ACTGAATGAC     840
TTGTGTTTCC TAAAGAGACT ATTACAAGAG ATAAAAACTT GTTGGAATAA AATTTTGATG     900
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TCCTCCAAGA ATCTATCTGC TTATGCAGTT TTTCAGAGTG GAATGCTTCC TAGAAGTTAC    1020
TGAATGCACC ATGGTCAAAA CGGATTAGGG CATTTGAGAA ATGCATATTG TATTACTAGA    1080
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TCAGTTGCAA TAATACTTCT CAAACCTGTT TAAATATTTC AAGACATTAA ATCTATGAAG    1260
TATATAATGG TTTCAAAGAT TCAAAATTGA CATTGCTTTA CTGTCAAAAT AATTTTATGG    1320
CTCACTATGA ATCTATTATA CTGTATTAAG AGTGAAAATT GTCTTCTTCT GTGCTGGAGA    1380
TGTTTTAGAG TTAACAATGA TATATGGATA ATGCCGGTGA GAATAAGAGA GTCATAAACC    1440
TTAAGTAAGC AACAGCATAA CAAGGTCCAA GATACCTAAA AGAGATTTCA AGAGATTTAA    1500
TTAATCATGA ATGTGTAACA CAGTGCCTTC AATAAATGGT ATAGCAAATG TTTTGACATG    1560
AAAAAAGGAC AATTTCAAAA AAATAAAAT                                      1589
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(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1585 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

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CACACCCTGA CAAGCTGCCA GGCAGGTTCT CTTCCTCTCA CATACTGACC CACGGCTCCA   60
CCCTCTCTCC CCTGGAAAGG ACACCATGAG CACTGAAAGC ATGATCCGGG ACGTGGAGCT  120
GGCCGAGGAG GCGCTCCCCA AGAAGACAGG GGGGCCCCAG GGCTCCAGGC GGTGCTTGTT  180
CCTCAGCCTC TTCTCCTTCC TGATCGTGGC AGGCGCCACC ACGCTCTTCT GCCTGCTGCA  240
CTTTGGAGTG ATCGGCCCCC AGAGGGAAGA GTCCCCCAGG GACCTCTCTC TAATCAGCCC  300
TCTGGCCCAG GCAGTCAGAT CATCTTCTCG AACCCCGAGT GACAAGCCTG TAGCCCATGT  360
TGTAGCAAAC CCTCAAGCTG AGGGGCAGCT CCAGTGGCTG AACCGCCGGG CCAATGCCCT  420
CCTGGCCAAT GGCGTGGAGC TGAGAGATAA CCAGCTGGTG GTGCCATCAG AGGGCCTGTA  480
CCTCATCTAC TCCCAGGTCC TCTTCAAGGG CCAAGGCTGC CCCTCCACCC ATGTGCTCCT  540
CACCCACACC ATCAGCCGCA TCGCCGTCTC CTACCAGACC AAGGTCAACC TCCTCTCTGC  600
CATCAAGAGC CCCTGCCAGA GGGAGACCCC AGAGGGGGCT GAGGCCAAGC CCTGGTATGA  660
GCCCATCTAT CTGGGAGGGG TCTTCCAGCT GGAGAAGGGT GACCGACTCA GCGCTGAGAT  720
CAATCGGCCC GACTATCTCG ACTTTGCCGA GTCTGGGCAG GTCTACTTTG GGATCATTGC  780
CCTGTGAGGA GGACGAACAT CCAACCTTCC CAAACGCCTC CCCTGCCCCA ATCCCTTTAT  840
TACCCCCTCC TTCAGACACC CTCAACCTCT TCTGGCTCAA AAAGAGAATT GGGGGCTTAG  900
GGTCGGAACC CAAGCTTAGA ACTTTAAGCA ACAAGACCAC CACTTCGAAA CCTGGGATTC  960
AGGAATGTGT GGCCTGCACA GTGAAGTGCT GGCAACCACT AAGAATTCAA ACTGGGGCCT 1020
CCAGAACTCA CTGGGGCCTA CAGCTTTGAT CCCTGACATC TGGAATCTGG AGACCAGGGA 1080
GCCTTTGGTT CTGGCCAGAA TGCTGCAGGA CTTGAGAAGA CCTCACCTAG AAATTGACAC 1140
AAGTGGACCT TAGGCCTTCC TCTCTCCAGA TGTTTCCAGA CTTCCTTGAG ACACGGAGCC 1200
CAGCCCTCCC CATGGAGCCA GCTCCCTCTA TTTATGTTTG CACTTGTGAT TATTTATTAT 1260
TTATTTATTA TTTATTTATT TACAGATGAA TGTATTTATT TGGGAGACCG GGGTATCCTG 1320
GGGGACCCAA TGTAGGAGCT GCCTTGGCTC AGACATGTTT TCCGTGAAAA CGGAGGCTGA 1380
ACAATAGGCT GTTCCCATGT AGCCCCCTGG CCTCTGTGCC TTCTTTTGAT TATGTTTTTT 1440
AAAATATTAT CTGATTAAGT TGTCTAAACA ATGCTGATTT GGTGACCAAC TGTCACTCAT 1500
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TGCTGAGGCC TCTGCTCCCC AGGGAGTTGT GTCTGTAATC GGCCTACTAT TCAGTGGCGA 1560

GAAATAAAGG TTGCTTAGGA AAGAA 1585

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

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(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CACTCGAGAA CTTTTTCTCC TTTAGATCAT ACAA 34

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CGGGAATTCA TGCCCCAGTT GACAACATAG 30

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

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(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

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(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

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(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GCGGCCGCGC ATGTACAGCA TGCAGCTCGC A 31

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GCGGCCGCTA AATAAATAGA GAGCCTTATG 30

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1011 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GGTTTATTTT CCAGATGCAA TCAATGCCCC AGTCACCTGC TGTTATAACT TCACCAATAG 60

GAAGATCTCA GTGCAGAGGC TCGCGAGCTA TAGAAGAATC ACCAGCAGCA AGTGTCCCAA 120

ACAAGCTGTG ATGTGAGTTC AGCACACCAA CCTTCCCTGG CCTGAAGTTC TTCCTTGTGG 180

AGCAAGGGAC AAGCCTCATA AACCTAGAGT CAGAGAGTGC ACTATTTAAC TTAATGTACA 240

AAGGTTCCCA ATGGGAAAAC TGAGGCACCA AGGGAAAAAG TGAACCCCAA CATCACTCTC 300

CACCTGGGTG CCTATTCAGA ACACCCAATT TCTTTAGCTT GAAGTCAGGA TGGCTCCACC 360

TGGACACCTA TAGGAGCAGT TTGCCCTGGG TTCCCTCCTT CCACCTGCGT TCCTCCTCTA 420

GCTCCCATGG CAGCCCTTTG GTGCAGAATG GGCTGCACTT CTAGACCAAA ACTGCAAAGG 480

AACTTCATCT AACTCTGTCC TCCCTCCCCA CAGCTTACAG ACCATTGTGG CAAGGAGATC 540

TGTGCTGACC CCAAGCAGAA GTGGGTTCAG GATTCCATGG ACCACCTGGA CAAGCAAACC 600

CAAACTCCGA AGACTTGAAC ACTCACTCCA CAACCCAAGA ATCTGCAGCT AACTTATTTT 660

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GAAACATTAT GCCTTAAGTA ATGTTAATTC TTATTTAAGT TATTGATGTT TTAAGTTTAT 780

CTTTCATGGT ACTAGTGTTT TTTAGATACA GAGACTTGGG GAAATTGCTT TTCCTCTTGA 840

ACCACAGTTC TACCCCTGGG ATGTTTTGAG GGTCTTTGCA AGAATCATTA ATACAAAGAA 900

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(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3194 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

AAGCTTGCTG AGAGTGGCTG CAGTCTCGCT GCTGGATGTG CACATGGTGG TCATTCCCTC 60

TGCTCACAGG GGCAGGGGTC CCCCCTTACT GGACTCAGGT TGCCCCCTGC TCCAGGTCCT 120

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TCAGGTAGTT CCCCCGCCTC CCTGGCATTT TGTGGTCACC ATTAATCATT TCCTCTGTGT 600

ATTTAAGAGC TCTTTTGCCA GTGAGCCCAG TACACAGAGA GAAAGGCTAA AGTTCTCTGG 660

AGGATGTGGC TGCAGAGCCT GCTGCTCTTG GGCACTGTGG CCTGCAGCAT CTCTGCACCC 720

GCCCGCTCGC CCAGCCCCAG CACGCAGCCC TGGGAGCATG TGAATGCCAT CCAGGAGGCC 780

CGGCGTCTCC TGAACCTGAG TAGAGACACT GCTGCTGAGA TGGTAAGTGA GAGAATGTGG 840

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GCACTCCTTG GGGAACACGT GGGTGGCAGC AGCGTCACCT GACCCAGGTC AGTGGGTGTG    2460
TCCTGGAGTG GGCCTCCTGG CCTCTGAGTT CTAAGAGGCA GTAGAGAAAC ATGCTGGTGC    2520
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CAGAAATCAG TAATATTTAT ATATTTATAT TTTTAAAATA TTTATTTATT TATTTATTTA 2940

AGTTCATATT CCATATTTAT TCAAGATGTT TTACCGTAAT AATTATTATT AAAAATATGC 3000

TTCTACTTGT CCAGTGTTCT AGTTTGTTTT TAACCATGAG CAAATGCCAG TGGGTGCCTG 3060

CCTTCCCATG AGGCAGGGGA GGGAGGAAAC GGGGAGGTGG AGAGGGGGCG GGGGCCTCCC 3120

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CATAGTCGGA ATTC 3194

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1491 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CCAAAGAAAA AGTGATTTGT CATTGCTTTA TAGACTGTAA GAAGAGAACA TCTCAGAAGT 60

GGAGTCTTAC CCTGAAATCA AAGGATTTAA AGAAAAAGTG GAATTTTTCT TCAGCAAGCT 120

GTGAAACTAA ATCCACAACC TTTGGAGACC CAGGAACACC CTCCAATCTC TGTGTGTTTT 180

GTAAACATCA CTGGAGGGTC TTCTACGTGA GCAATTGGAT TGTCATCAGC CCTGCCTGTT 240

TTGCACCTGG GAAGTGCCCT GGTCTTACTT GGGTCCAAAT TGTTGGCTTT CACTTTTGAC 300

CCTAAGCATC TGAAGCCATG GGCCACACAC GGAGGCAGGG AACATCACCA TCCAAGTGTC 360

CATACCTCAA TTTCTTTCAG CTCTTGGTGC TGGCTGGTCT TTCTCACTTC TGTTCAGGTG 420

TTATCCACGT GACCAAGGAA GTGAAAGAAG TGGCAACGCT GTCCTGTGGT CACAATGTTT 480

CTGTTGAAGA GCTGGCACAA ACTCGCATCT ACTGGCAAAA GGAGAAGAAA ATGGTGCTGA 540

CTATGATGTC TGGGGACATG AATATATGGC CCGAGTACAA GAACCGGACC ATCTTTGATA 600

TCACTAATAA CCTCTCCATT GTGATCCTGG CTCTGCGCCC ATCTGACGAG GGCACATACG 660

AGTGTGTTGT TCTGAAGTAT GAAAAAGACG CTTTCAAGCG GGAACACCTG GCTGAAGTGA 720

CGTTATCAGT CAAAGCTGAC TTCCCTACAC CTAGTATATC TGACTTTGAA ATTCCAACTT 780

CTAATATTAG AAGGATAATT TGCTCAACCT CTGGAGGTTT TCCAGAGCCT CACCTCTCCT 840

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ATGGAATTTT TGTGATATGC TGCCTGACCT ACTGCTTTGC CCCAAGATGC AGAGAGAGAA 1140

GGAGGAATGA GAGATTGAGA AGGGAAAGTG TACGCCCTGT ATAACAGTGT CCGCAGAAGC 1200

AAGGGGCTGA AAAGATCTGA AGGTAGCCTC CGTCATCTCT TCTGGGATAC ATGGATCGTG 1260

GGGATCATGA GGCATTCTTC CCTTAACAAA TTTAAGCTGT TTTACCCACT ACCTCACCTT 1320

CTTAAAAACC TCTTTCAGAT TAAGCTGAAC AGTTACAAGA TGGCTGGCAT CCCTCTCCTT 1380

TCTCCCCATA TGCAATTTGC TTAATGTAAC CTCTTCTTTT GCCATGTTTC CATTCTGCCA 1440

TCTTGAATTG TCTTGTCAGC CAATTCATTA TCTATTAAAC ACTAATTTGA G 1491

CLAIMS

1. A DNA construct comprising (i) means for expression of a coding sequence in a tumour cell and (ii) a said coding sequence encoding a cytokine.

2. A construct according to Claim 1 wherein the said means for expression provides for specific expression selectively in tumour cells.

3. A construct according to Claim 2 wherein the tumour cells are melanoma cells.

4. A construct according to Claim 2 wherein the tumour cells are breast tumour cells.

5. A construct according to Claim 2 wherein the tumour cells are colon tumour cells.

6. A construct according to Claim 2 wherein the tumour cells are pancreatic tumour cells.

7. A construct according to Claim 2 wherein the tumour cells are prostate tumour cells.

8. A construct according to Claim 3 wherein the said means for expression is a promoter or an analogue or part thereof forming part of a gene expressed exclusively in the melanin synthesis pathway.

9. A construct according to Claim 8 wherein the gene is tyrosinase or TRP-1.

10. A construct according to Claim 4 wherein the said means for expression is provided by the c-*erb*-B2 gene promoter or the MUC1 gene promoter or the c-*erb*-B3 gene promoter.

11. A construct according to Claim 5 wherein the said means for expression is provided by the CEA gene promoter.

12. A construct according to Claim 6 wherein the said means for expression is provided by the MUC1 gene promoter.

13. A construct according to Claim 7 wherein the said means for expression is provided by the PSA gene promoter.

14. A construct according to any one of the preceding claims wherein the cytokine is interleukin-2 or interleukin-4.

15. A construct according to any one of the preceding claims further comprising a B7 coding sequence and means for expression thereof in a tumour cell.

16. A composition comprising a construct according to any one of the preceding claims and means for selectively delivering it to a tumour.

17. A composition according to Claim 16 wherein the selective delivery means is a liposome carrying tumour cell targeting means or a retrovirus or adenovirus specific for the tumour cells.

18. A method of treating a tumour and/or ameliorating metastasis therefrom comprising delivering into cells of the tumour a construct according to any one of Claims 1 to 15.

19. A method of treating a tumour and/or ameliorating metastasis therefrom comprising delivering into cells of the tumour a construct according to any one of Claims 1 to 15 expressing at least two coding sequences encoding respective cytokines wherein the said cytokines may be the same as or different from one another.

20. A method of treating a tumour and/or ameliorating metastasis therefrom comprising delivering into cells of the tumour a plurality of constructs according to any one of Claims 1 to 15 expressing at least two coding sequences encoding respective cytokines wherein the said cytokines may be the same as or different from one another.

21. A method according to Claims 19 or 20 wherein the cytokines are chosen from interleukin-2, interleukin-4, macrophage colony stimulating factor, interferon-γ, tumour necrosis factor and interleukin-7.

22. A method according to Claims 19 or 20 wherein the coding sequences encode interleukin-2, interleukin-4 and macrophage colony stimulating factor and are present in 1:1:1 molar ratio.

23. A method according to any one of Claims 18 to 20 wherein the tumour cells are melanoma, breast, pancreas, prostate or colon cells and naked DNA is injected directly into the tumour.

24. A method according to any one of Claims 18 to 23 additionally comprising administering a chemotherapeutic agent.

25. A method according to Claim 24 wherein the chemotherapeutic agent is at least one of cisplatin, dacarbazine, tamoxifen, nitrosourea, vinca alkaloid, melphalan, doxorubicin, adriamycin, etoposide and 5-fluorouracil.

26. A method according to any one of Claims 18 to 25 further comprising delivering into cells of the tumour a construct comprising a B7 coding region and means for expression thereof in a tumour cell.

27. A method according to any one of Claims 18 to 25 comprising delivering into cells of the tumour a construct comprising a B7 coding region and a cytokine coding region and means for expression thereof in a tumour cell.

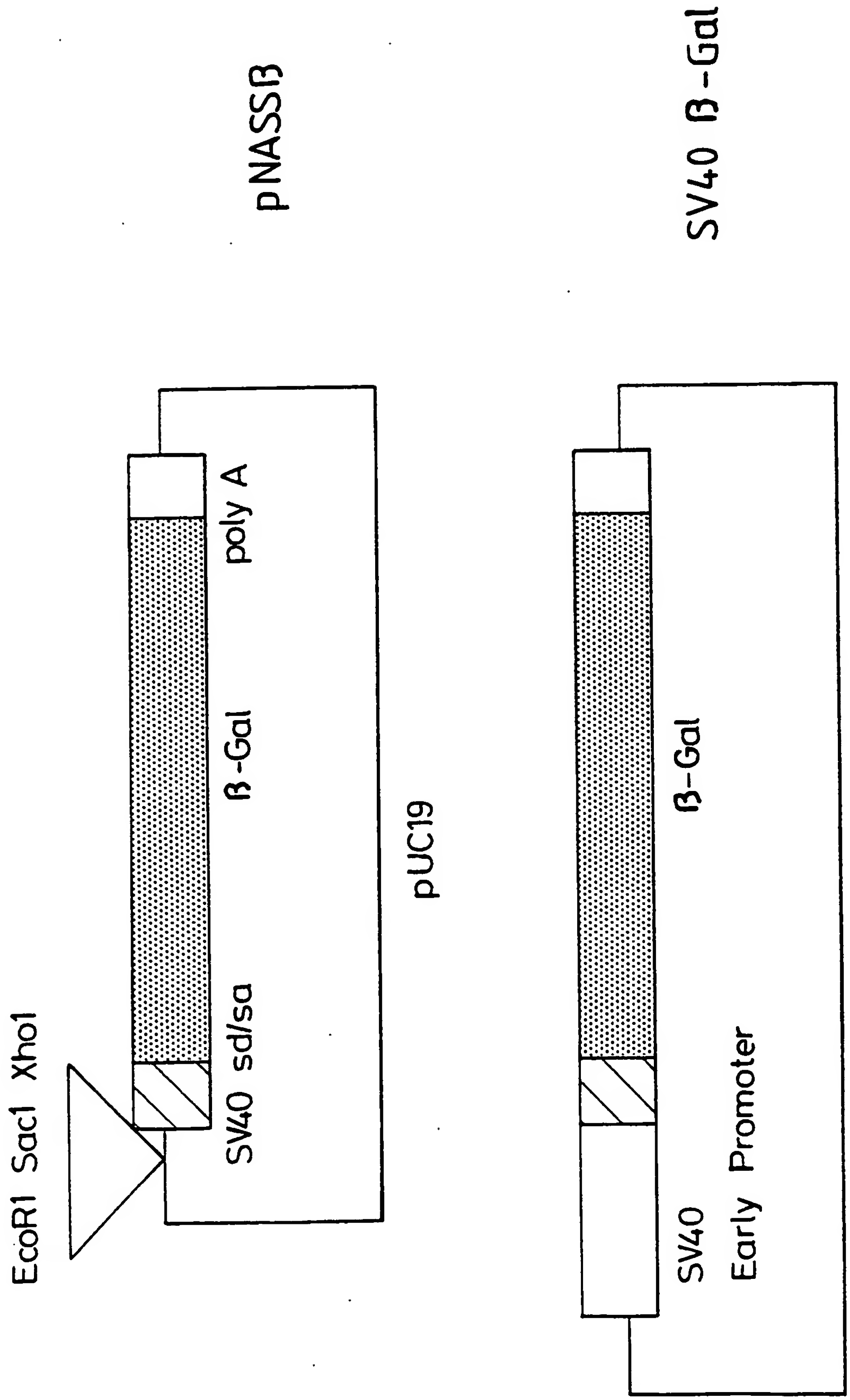


Fig. 1 (PAGE 1 of 3)

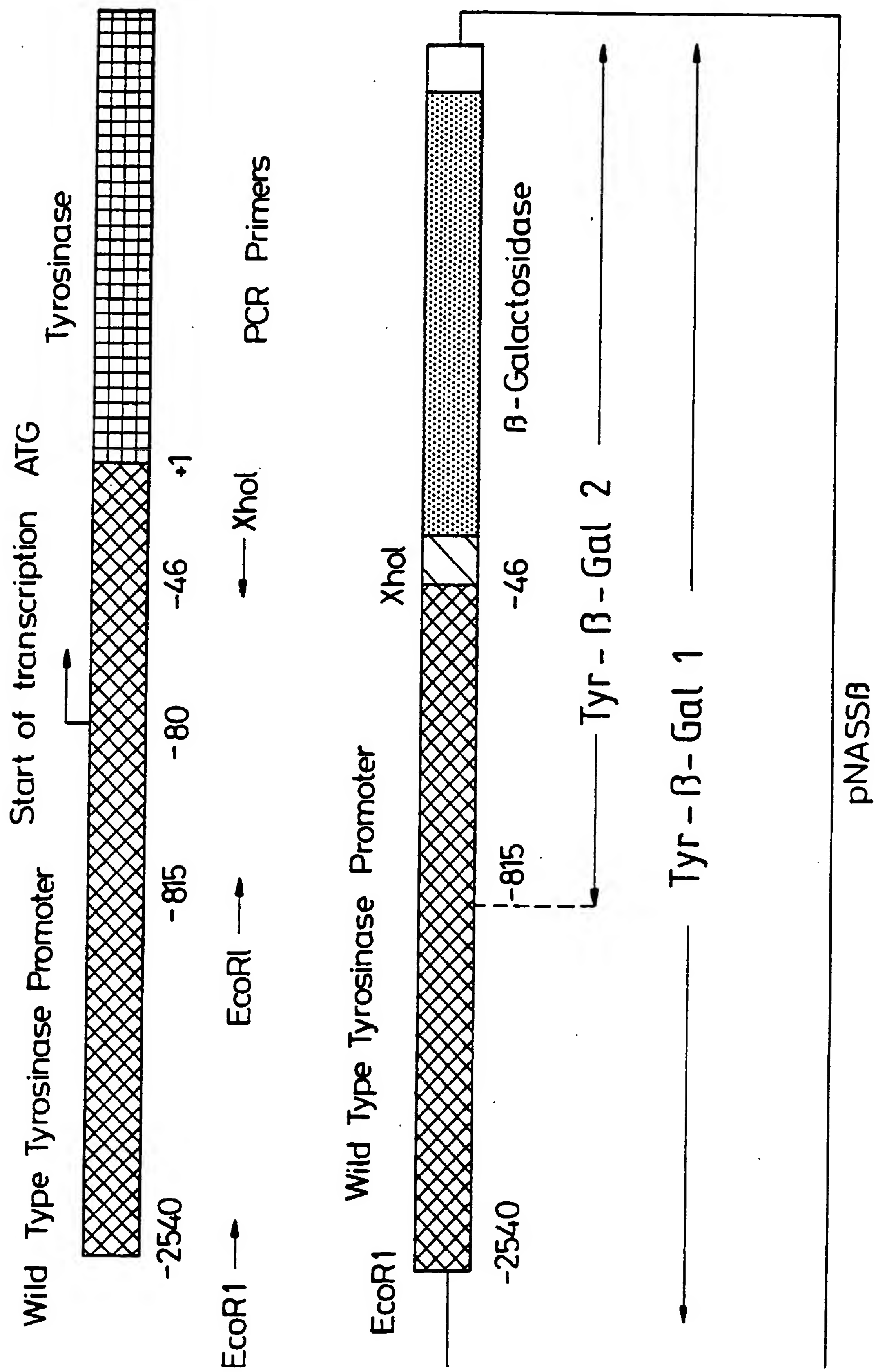


Fig. 1 (PAGE 2 of 3)

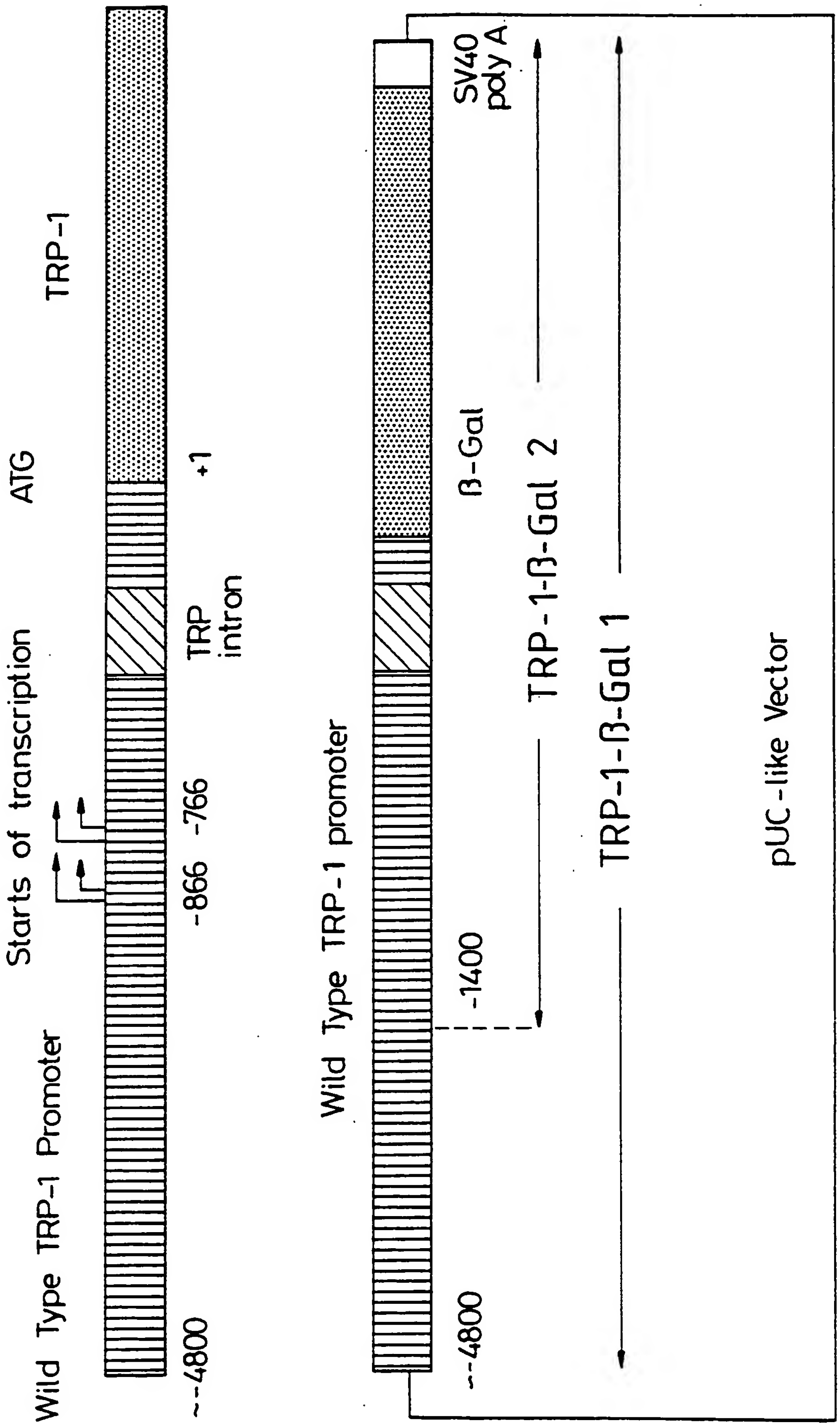
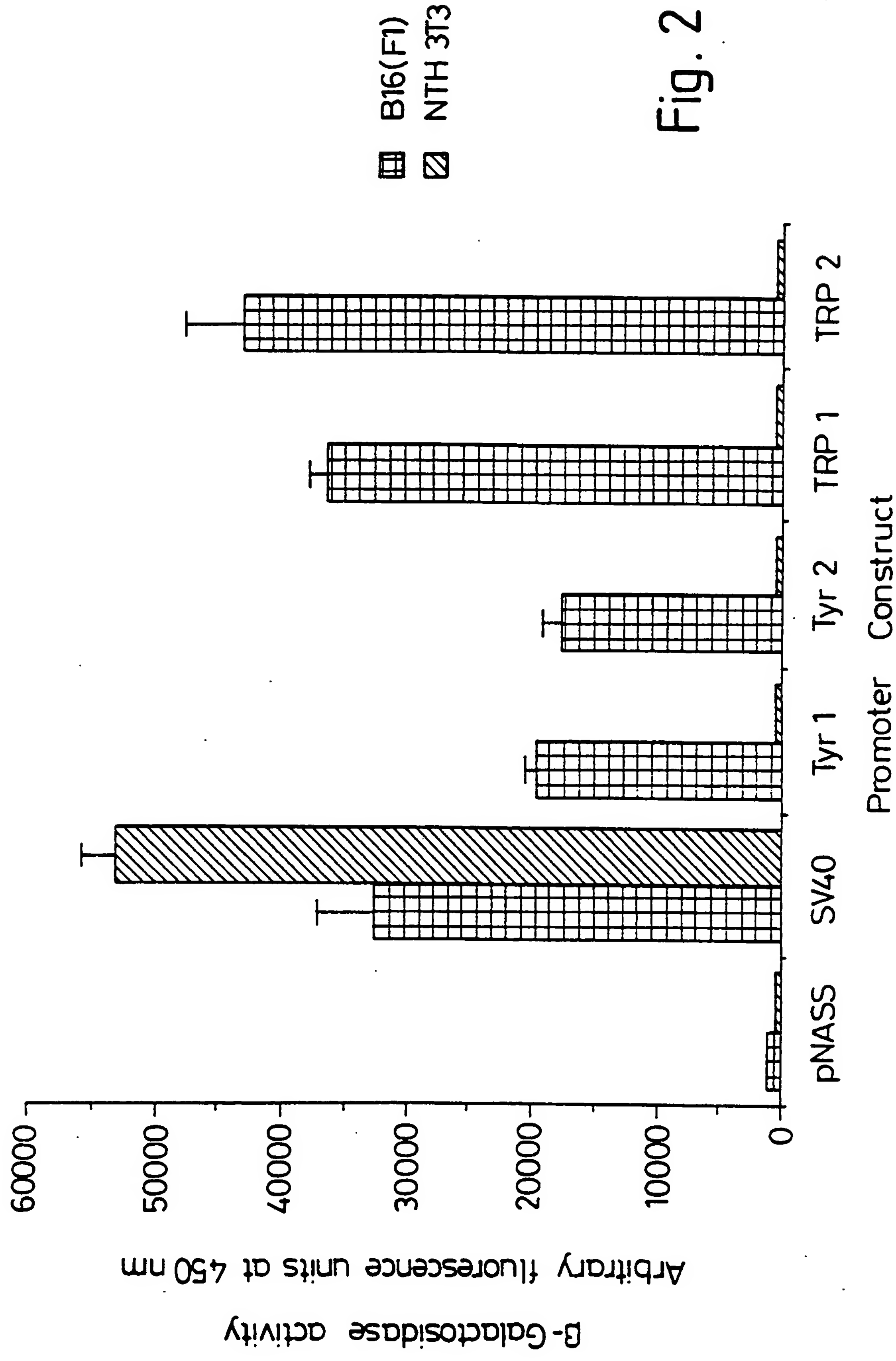


Fig. 1 (PAGE 3 of 3)


B16(F1)
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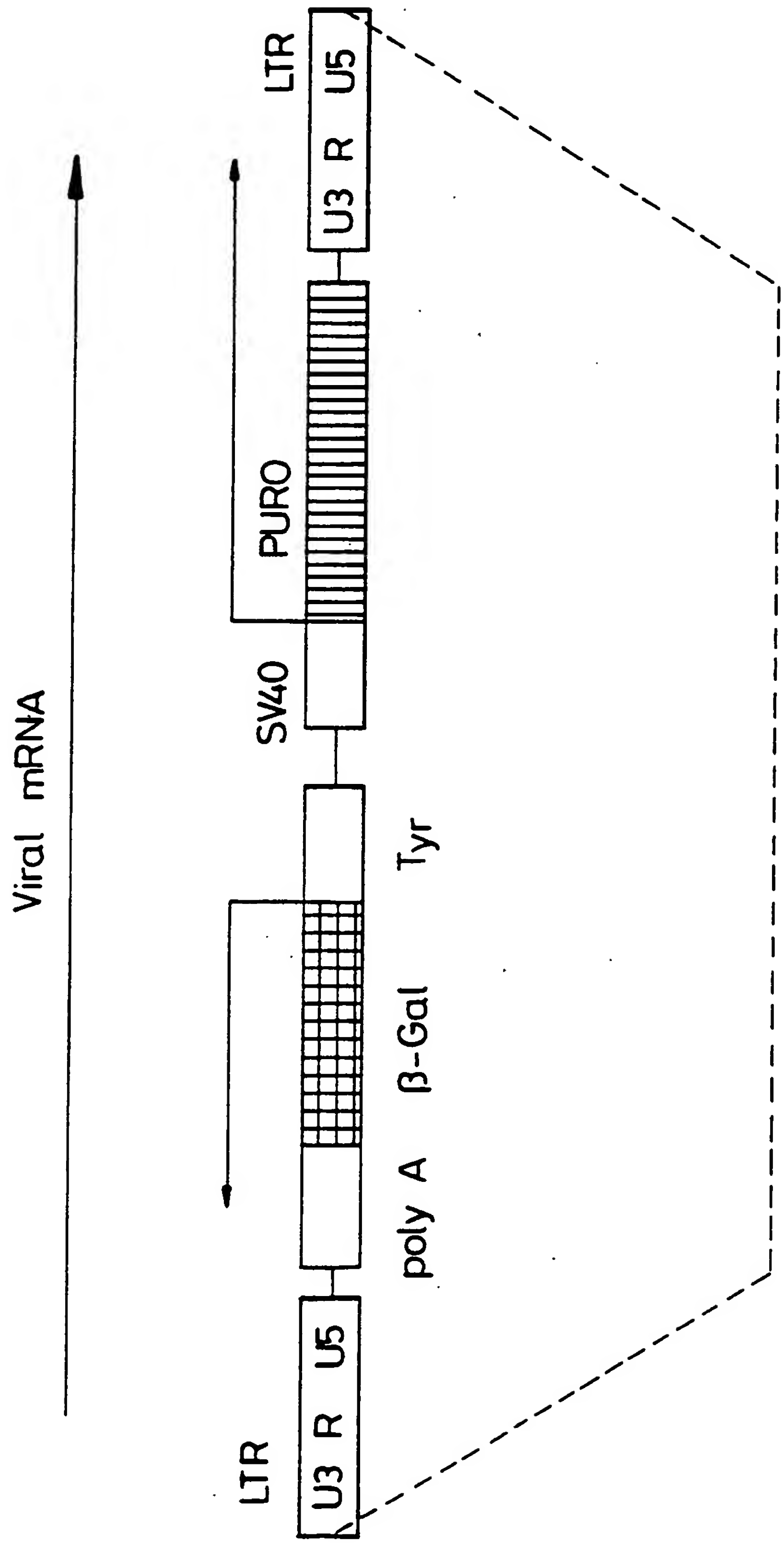
60000
50000
40000
30000
20000
10000
0
Arbitrary fluorescence units at 450 nm
β-Galactosidase activity
pNASS
SV40
Tyr 1
Tyr 2
TRP 1
TRP 2
Promoter Construct

Fig. 2



pBabe Puro (Tyr-β-Gal)

Fig. 3

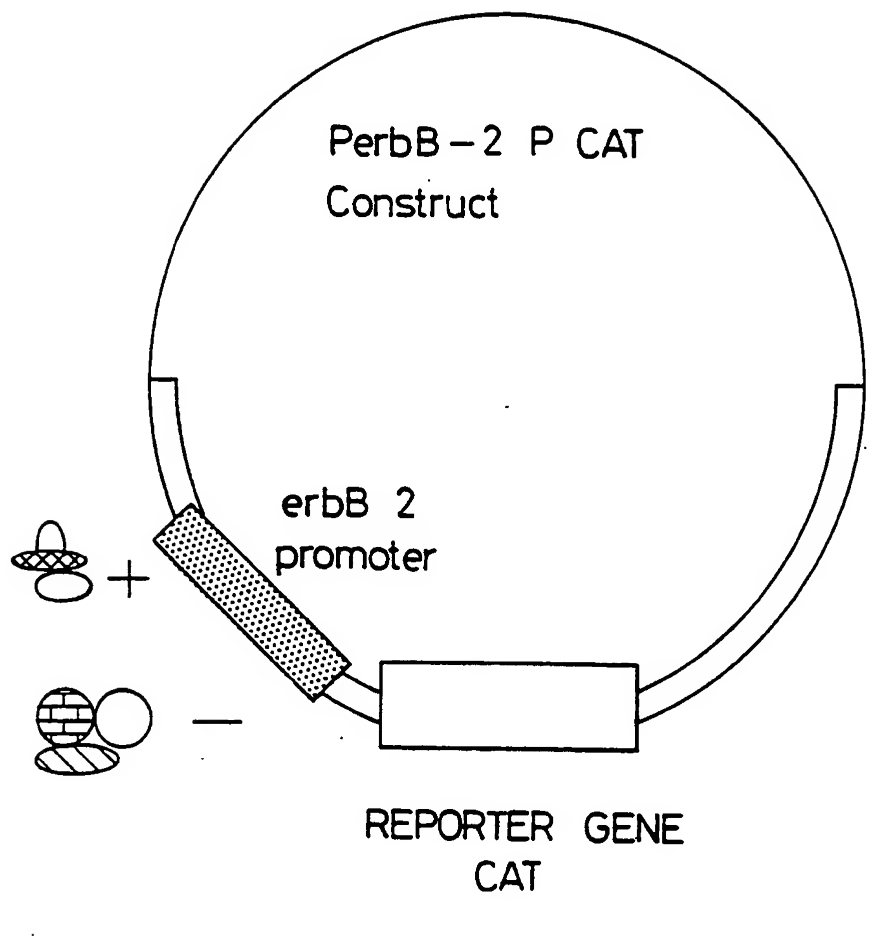
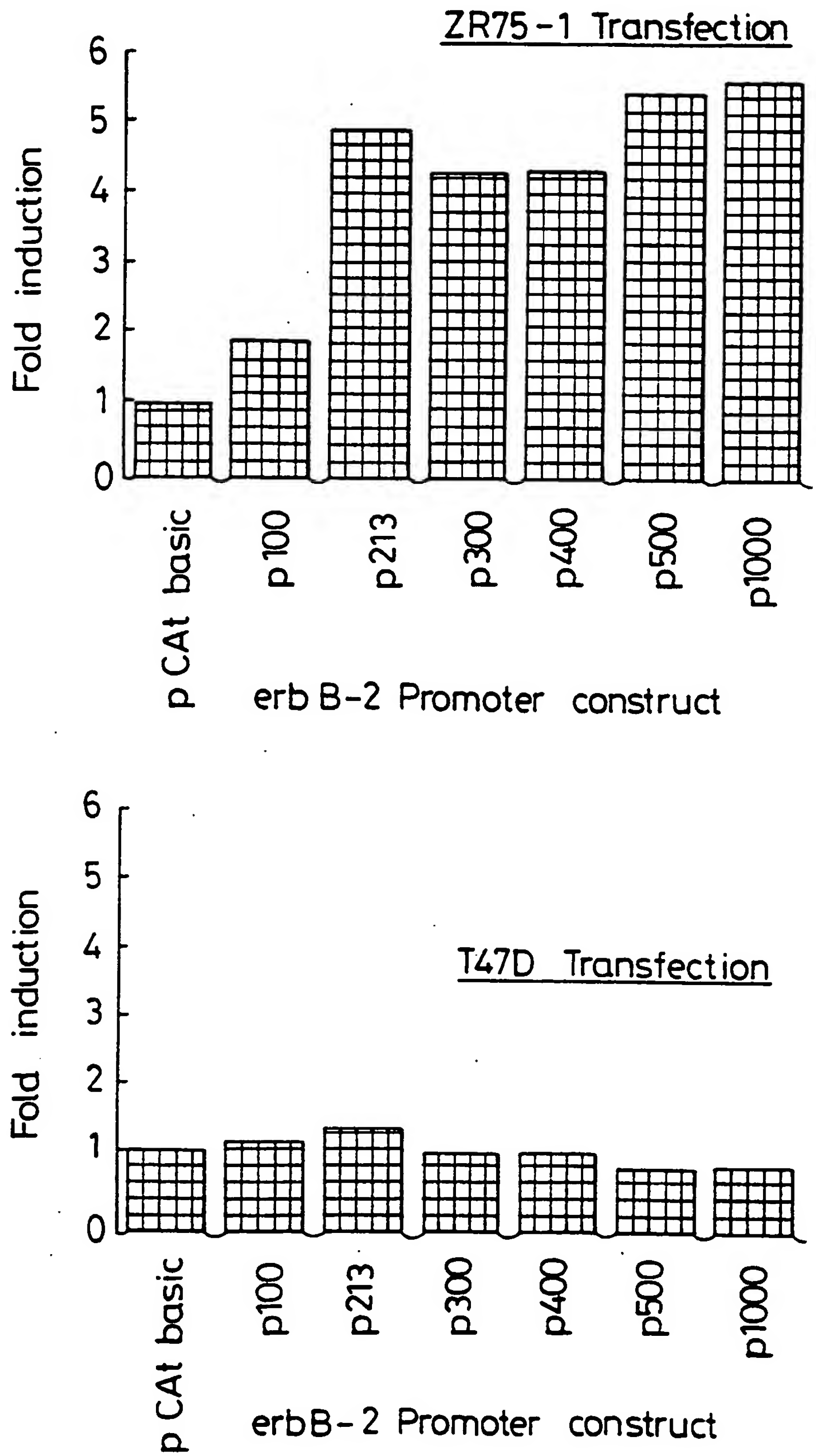


Fig. 4


ZR75-1 Transfection
Fold induction
0
1
2
3
4
5
6
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p213
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p400
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p1000
erb B-2 Promoter construct
T47D Transfection
Fold induction
0
1
2
3
4
5
6
p CAt basic
p100
p213
p300
p400
p500
p1000
erbB-2 Promoter construct

Fig. 5

GC Box

-787 CGAGCGGCCCCTCAGCTTCGGCGCCCAGCCCCGCAAGGCTCCCGGTGACCACTAGAGGGCGGGAGGAGCTCCTGGCCAGT

-707 GGTGGAGAGTGGCAAGGAAGGACCCTAGGGTTCATCGGAGCCCAGGTTTACTCCCTTAAGTGGAAATTTCTTCCCCCACT

Sp1

-627 CCTCCTTGGCTTTCTCCAAGGAGGGAACCCAGGCTGCTGGAAAGTCCGGCTGGGGCGGGGACTGTGGGTTCAGGGGAGAA

MPBF

-547 CGGGGTGTGGAACGGGACAGGGAGCGGTTAGAAGGGTGGGGCTATTCCGGGAAGTGGTGGGGGGAGGGAGCCCAAAACTA

GC Box

-467 GCACCTAGTCCACTCATTATCCAGCCCTCTTATTTCTCGGCCGCTCTGCTTCAGTGGACCCGGGGAGGGCGGGGAAGTGG

-387 AGTGGGAGACCTAGGGGTGGGCTTCCCGACCTTGCTGTACAGGACCTCGACCTAGCTGGCTTTGTTCCCCATCCCCACGT

-307 TAGTTGTTGCCCTGAGGCTAAAACTAGAGCCCAGGGGCCCCAAGTTCCAGACTGCCCCTCCCCCCTCCCCCGGAGCCAGG

-227 GAGTGGTTGGTGAAAGGGGGAGGCCAGCTGGAGAACAAACGGGTAGTCAGGGGGTTGAGCGATTAGAGCCCTTGTACCCT

Sp1 E-MUC1

-147 ACCCAGGAATGGTTGGGGAGGAGGAGGAAGAGGTAGGAGGTAGGGGAGGGGGCGGGGTTTTGTCACCTGTCACCTGCTCG

GC Box +1

-67 CTGTGCCTAGGGCGGGCGGGCGGGGAGTGGGGGGACCGGTATAAAGCGGTAGGCGCCTGTGCCCGCTCCACCTCTCAAGC

+14 AGCCAGCGCCTGCCTGAATCTGTTCTGCCCCCTCCCCACCCATTTCACCACCACCATG

+33

Fig. 6

```
         NcoI
         |
-1571    CATGGTGTCCGACTTATGCCCGAGAAGATGTTGAGCAAACTTATCGCTTATCTGCTTCTCATAGAGTCTT
-1501    GCAGACAAACTGCGCAACTCGTGAAAGGTAGGCGGATCTGGGTCGACCTGCAGGTCAACGGATCCCTTCT
-1431    TGACCAGTATAGCTGCATTCTTGGCTGGGGCATTCCAACTAGAACTGCCAAATTTAGCACATAAAAATAA
-1361    GGAGGCCCAGTTAAATTTGAATTTCAGATAAACAATGAATAATTTGTTAGTATAAATATGTCCCATGCAA
-1291    TATCTTGTTGAAATTAAAAAAAAAAGTCTTCCTTCCATGCCCCACCCCTACCACTAGGCCTAAGGAATAG
-1221    GGTCAGGGGCTCCAAATAGAATGTGGTTGAGAAGTGGAATTAAGCAGGCTAATAGAAGGCAAGGGGCAAA
-1151    GAAGAAACCTTGAATGCATTGGGTGCTGGGTGCCTCCTTAAATAAGCAAGAAGGGTGCATTTTGAAGAAT
-1081    TGAGATAGAAGTCTTTTTGGGCTGGGTGCAGTTGCTCGTGGTTGTAATTCCAGCACTTTGGGAGGCTGAG
-1011    GCGGGAGGATCACCTGAGGTTGGGAGTTCAAGACCAGCCTCACCAACGTGGAGAACCCTGTCTTTACTAA
 -941    AAATACAAAAAATTCAGCTGGTCATGGTGGCACATGCCTGTAATCCCAGCTGCTCGGGAGGCTGAGGCAG
 -871    GAGAATCACTTGAACCAGGGAGGCAGAGGTTGTGGTGAGCAGAGATCGCGCCATTGCTCTCCAGCCTGGG
 -801    CAACAAGAGCAAAAGTTCGTTTAAAAAAAAAAAAAAGTCCTTTCGATGTGACTGTCTCCTCCCAAATTTG
 -731    TAGACCCTCTTAAGATCATGCTTTTCAGATACTTCAAAGATTCCAGAAGATATGCCCCGGGGTCCTGGA
 -661    AGCCACAAGGTAAACACAACACATCCCCCTCCTTGACTATCAATTTTACTAGAGGATGTGGTGGAAAAC
 -591    CATTATTTGATATTAAAACAAATAGGCTTGGGATGGAGTAGGATGCAAGCTCCCCAGGAAAGTTTAAGAT
 -521    AAAACCTGAGACTTAAAAGGGTGTTAAGAGTGGCAGCCTAGGGAATTTATCCCGGACTCCGGGGGAGGGG
 -451    GCAGAGTCACCAGCCTCTGCATTTAGGGATTCTCCGAGGAAAGTGTGAGAACGGCTGCAGGCAACCCAG
```

FIG. 7 (PAGE 1 OF 2)

```
-381   GCGTCCCGGCGCTAGGAGGGACGACCCAGGCCTGCGCGAAGAGAGGGAGAAAGTGAAGCTGGGAGTTGCC
-311   GACTCCCAGACTTCGTTGGAATGCAGTTGGAGGGGGCGAGCTGGGAGCGCGCTTGCTCCCAATCACAGGA
-241   GAAGGAGGAGGTGGAGGAGGAGGGCTGCTTGAGGAAGTATAAGAATGAAGTTGTGAAGCTGAGATTCCCC
-171   TCCATTGGGACCGGAGAAACCAGGGGAGCCCCCCGGGCAGCCGCGCGCCCCTTCCCACGGGGCCCTTTAC
-101   TGCGCCGCGCGCCCGGCCCCCACCCCTCGCAGCACCCCGCGCCCCGCGCCCTCCCAGCCGGGTCCAGCCG
-31    GAGCCATGGGGCCGGAGCCGCAGTGAGCACCATGGAGCTGG
             |                        -1
           NcoI
```

FIG. 7 (PAGE 2 OF 2)

C erb B3 promoter

```
BamHI    SmaI           FP/A                  FP/B
GGATCCGTCCCGGGACTAGCAGGGCTTTGGGCAGCAACCCGCAGGAGCCCGACCGCCTCTGGCCAGGTCC
1                                                     OB2-1           70
        FP/C
GGGCAGCTGGTGGGGGAGGTTCCAGAGGTCCACGCCATTCGTGGACGCAGTCTCTAGTGTCCTCTCCGCG
71                                                                   140

TCCCACTTCACTGCCCCATCCCCTTTCCTGCGAGAGCCTGGACTTGGAAGGCACCTGGGAGGGTGTAAGC
141                                                                  210

GCCTTGGTGTGTGCCCATCTGGGTCCCCAGAAGAGCGGCGGGAACTGCGGCCGCCCGGACGGTGCGGCCA
211                                                                  280

GACTCCAGTGTGGAAGGGGAGGCAGCTGTTCTCCCAGGCGGCCGTGGGGGCAGCAGAGGGGACGGCGAC
281                                                                  350
```

Fig. 8 (PAGE 1 of 3)

```
AGGTGCGGGAGCCCCTCCCGGGGTAGAAGTGGAAAGGCGGGCTCCGGGGTCTGTTCCCAGGCTGGAAACC
351                                                                 420
                  SmaI
ACCCCCGCCCCCCATCCAAATCCCCGGGAGAGGCCCGGCCGGCGCCGGGTCTGGAGGAGGAAGCGGCCAG
421                                                                 490

AGACAGTGCAATTTCACGCGGTCTCTGTGGCTCGGGTTCCTGGGCTGGGTGGATGAATTATGGGGTTTCG
491                                                                 560

AGTCTGGGAGAAACTGAGGTGGCCTGGACGTGAGGCAAAAACACCCTCCCCCTCAAAAACACACAGAGA
561                                                                 630
                          FP/D
GAAATATTCACATTCTGAGAGAAAATCCACCAAGTGAACCAACCGGCTAGGGGAGTTGAGTGATTTGGTT
631                                                                 700
                               FP/E
AATGGGCGAGGCCAACTTTCAGGGGGCAGGGCTTTGGAGAGCTTTCCACTCCCTCATTCATTACCCTTCC
701                                                                 770
                                      PstI
CTGGATCTGGGGGCTTTCGGAATCTCGACCTCCCCTTGGCCTATCTCCTGCAGAAAAATTAGGGTGAGCC
771                                                                 840

CCATCCTCGATCTGCTCCGCCAAGTTGCGGGACCGCGGGGCGTGGCACGCTCAGGGGCAGGCGGTCCGAG
841                                                                 910
```

Fig. 8 (PAGE 2 of 3)

GCTCCGCAATCCCCACTCCAGCCTCGCGCGGAGGGGCGCGGCCCGTGTGACTCACCCCCTTCCCTCTG
911 980

CGTTCCTCCCTCCCTCTCTCTCTCTCTCACACACACACCCCTCCCCTGCCATCCCTCCCCGGACTC
981 P 1050

CGGCTCCGGCTCCGATTGCAATTTGCAACCTCCGCTGCCGTCGCCGCAGCAGCCACCAATTCGCCAGCGG
1051 +1 K 1120

SmaI
TTCAGGTGGCTCTTGCCTCGATGTCCTAGCCTAGGGGCCCCCGGGCCGGACTTGGCTGGGCTCCCTTCAC
1121 1190

PstI SmaI
CCTCTGCGGAGTCATGAGGGCGAACGACGCTCTGCAGGTGCTGGGCTTGCTTTTCAGCCTGGCCCGGGGC
1191 METArgAlaAsnAspAlaLeuGlnValLeuGlyLeuLeuPheSerLeuAlaArgGly
1260

1st intron
TCCGAGGTGGGCAACTCTCAGGCAGGTAAGTGCCCAGAGAGCACC
SerGluValGlyAsnSerGlnAla 1305
1261

Fig. 8 (PAGE 3 of 3)

INTERNATIONAL SEARCH REPORT

Interr al Application No
PCT/GB 93/01730

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K48/00 C12N15/86 C12N15/26 C12N15/85 A61K33/24
A61K31/70 A61K31/71 //(A61K33/24,31:71,31:70,31:505,31:475,
31:415,31:195,31:17,31:135),C12N15/24,C12N15/27,C12N15/28,

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CANCER RESEARCH vol. 53 , 1 March 1993 pages 962 - 967 R. G. VILE ET AL 'In vitro and in vivo targeting of gene expression to melanoma cells' see the whole document see especially page 966 right column --- -/--	1-3,8,9, 16-18,23

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

3

Date of the actual completion of the international search	Date of mailing of the international search report
30 November 1993	16. 12. 93

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Le Cornec, N

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 93/01730

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/23
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NUCLEIC ACIDS RESEARCH. vol. 19, no. 14, 1991, ARLINGTON, VIRGINIA US pages 3799 - 3804 I. J. JACKSON ET AL 'The Tyrosinase-related protein-1 gene has a structure and promoter sequence very different from Tyrosinase' cited in the application see the whole document especially the abstract ,page 3802 left column line 9 -right column line 4 , page 3803 right column ---	1-3,8,9, 16-18,23

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

3

Date of the actual completion of the international search	Date of mailing of the international search report
30 November 1993	5. 12. 93
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Le Cornec, N

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88 , September 1991 , WASHINGTON US pages 8039 - 8043 B. E. HUBER ET AL 'Retroviral-mediated gene therapy for the treatment of hepatocellukar carcinoma : A innovative approach for cancer therapy' see the whole document especially page 8039 ---	1-3,8,9, 16-18,23
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 88 , January 1991 , WASHINGTON US pages 164 - 168 M. BRADL ET AL 'Malignant melanoma in transgenic mice' cited in the application see page 164 see page 167, right column, line 21 - line 39 ---	1-3,8,9, 16-18,23
A	ANNALS OF PASTIC SURGERY vol. 28, no. 1 , January 1992 pages 114 - 118 M. SIVANANDHAM ET AL 'Prospects for gene therapy and lymphokine therapy for metastatic melanoma' ---	
X	CELL vol. 60, no. 3 , 9 February 1990 , CAMBRIDGE, NA US pages 397 - 403 E. R. FEARON ET AL 'Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response' cited in the application see the whole document especially page 400 right column, table 2 and pages 401-402 ---	1,14, 18-21, 234
Y	EP,A,0 415 731 (THE WELLCOME FOUNDATION LIMITED) 6 March 1991 see the whole document ---	1-3,8,9, 16-18
X	JOURNAL OF IMMUNOLOGY vol. 146, no. 9 , 1 May 1991 , BALTIMORE US pages 3227 - 3234 A.L. ASHER ET AL 'Murine tumor cells transduced with the gene for tumor necrosis factor-alpha' see the whole document ---	1,18

-/--

3

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IMMUNOLOGY TODAY vol. 11, no. 6 , 1990 , CAMBRIDGE GB pages 196 - 200 S.J. RUSSEL 'Lymphokine gene therapy for cancer' see the whole document ---	1,16
T	NATURE vol. 357 , 11 June 1992 , LONDON GB pages 455 - 460 A. DUSTY MILLER ET AL 'Human gene therapy comes of age' -----	

3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB93/01730

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 18-27 (as far as they concern in vivo methods) are directed to the treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 93/01730

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0415731	06-03-91	AU-A- 6199190	07-03-91
		CN-A- 1050899	24-04-91
		JP-A- 3172189	25-07-91